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CIGARETTE SMOKING, LIPOLYSIS AND VASCULAR DISEASE

by

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TABLE OF CONTENTS

LIST OF TABLES

Page No.

Page No.

Page No.

LIST OF FIGURES

Page No.

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SUMMARY

This thesis presents clinical and experimental studies relating to the effect of cigarette smoking on lipolysis in subjects with various types of vascular disease.

The introduction reviews the evidence of the harmful effects of cigarette smoking and for its possible contribution to vascular disease through its action on lipid metabolism via the sympathetic adreno-medullary systems.

The aims of the study are then outlined with respect to the effect of smoking varying numbers of cigarettes on levels of free fatty acids (FFA), ketone bodies (aceto-acetate and B hydroxybutyrate), glucose, insulin, cholesterol and triglyceride in subjects with either stable coronary heart disease (CHD) as manifest by angina or with peripheral vascular disease (PVD).

The standard situation studied was serial venous sampling under controlled conditions before and after the smoking of two normal nicotine content cigarettes. Initial investigations indicated that a satisfactory steady state was achieved prior to smoking.

Basal FFA values of the recent infarction group were consistently higher than those found in other subjects and suggested a possible residual "stress" situation. Following cigarette smoking the increments in FFA values were significantly higher in the recent infarction subjects. Changes in FFA were paralleled by increased ketone body levels. The findings indicated that the

increased response to cigarette smoking was a feature of recent myocardial infarction rather than occurring in all types of clinical vascular disease. In no group was there any evidence to support the suggestion made by others that cigarette smoking might affect insulin, cholesterol or triglyceride levels. These variables remained unaltered. The subcutaneous injection of 0.01 ul of 1:10,000 adrenaline per kg bodyweight corresponded to the effect of cigarette smoking. Increased levels of FFA occurred in the post-infarction group again perhaps indicating an increased response to sympathetic or adrenomedullary activity.

The effect of cigarette smoking was also examined in subjects classified according to their lipoprotein status (normal lipids, Type II or Type IV hyperlipoproteinaemia). Overall percentage changes in FFA and ketone bodies were comparable in each group although the absolute changes in FFA were greater in Type IV subjects.

Similar studies were done in subjects one year after myocardial infarction. The FFA and ketone body increases that developed were similar to those of control subjects and considerably less than those found 3 weeks after infarction. This suggested that the enhanced response to cigarette smoking is a temporary rather than a persistent finding. In addition, it was found that the increased FFA levels following smoking could be abolished by the prior administration of the beta-adrenoreceptor blocking agent propranolol. This effect could have important

clinical implications for the long-term management of patients with CHD.

Studies were also undertaken on the effect of smoking 6 normal cigarettes over 3 hours. The response in terms of FFA and ketone body increases (40% and 100% respectively) was similar in control subjects and those with angina or PVD. There was no evidence of an enhanced response among those subjects with vascular disease. There was, however, considerable variation among subjects in terms of blood glucose levels before and after smoking. The changes in glucose could not, however, be correlated with either changes in FFA or insulin levels.

Measurement of individual fatty acid levels before and after smoking were done using the combination of thin layer and gas-liquid chromatography. There appeared to be a general increase in FFA levels after smoking rather than a change in only certain individual fatty acids. Nor did there appear to be any alteration in the fatty acid component of the triglyceride moiety.

A series of investigations were done on glycerol kinetics in control subjects and those with vascular disease. The rate of removal of intravenously injected glycerol (50 ml of 10% glycerol) could be expressed as a single exponential with T½ values being similar in all groups studied. In addition, it was found that cigarette smoking following the administration of glycerol did not produce any alteration in the rate of removal from the bloodstream.

An unexpected finding following glycerol infusion was an apparent rise in FFA levels, when measured by the Dole method. Detailed investigation by colorimetric and gas-liquid chromatographic methods indicated that in fact glycerol infusion resulted in a marked decrease in plasma FFA. It seems likely that infusion of glycerol results in the accumulation in the plasma of an acidic compound - as yet unknown - which is titrated by the Dole method as FFA.

THERE EXISTS A DOSE RESPONSE BETWEEN THE NUMBER OF CIGARETTES SMOKED AND OVERALL MORTALITY AND A SPECIFIC RELATIONSHIP TO DEATH FROM MYOCARDIAL INFARCTION AND LUNG CANCER.

NATIONAL CANCER INSTITUTE MONOGRAPH 28, JUNE 1968.
U.S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE.

IT IS ESTABLISHED THAT MALE CIGARETTE SMOKERS HAVE A HIGHER DEATH RATE FROM CORONARY DISEASE THAN NON-SMOKING MALES.

REPORT OF THE ADVISORY COMMITTEE TO THE SURGEON GENERAL OF THE PUBLIC HEALTH SERVICE.
U.S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE.

CHAPTER ONE - INTRODUCTION AND GENERAL REVIEW

The plant, Nicotinia Tabacum, is the chief source of smoking tobacco. Originating in America, it was introduced to Europe in 1560 by Jean Nicot, the French Ambassador to Portugal. He believed it had medicinal qualities, telling Catherine de Medici that "through the general use of tobacco Her Majesty's subjects would become easy to govern". James I and VI, held different views, stating in 1603 that "smoking is a habit disgusting to the eyes, odious to the nose and harmful to the lungs". It is difficult to be certain when physicians began to suspect tobacco as a cause of disease but according to Gelineau (1887)[38], Beau in 1862 was the first to show convincingly that tobacco smoking and angina pectoris could, under certain conditions, be associated.

Little was subsequently learned of the possible untoward effects of smoking until the 20th Century. In 1939, Muller[89] compared the smoking habits of 86 patients with lung cancer with those of an equal number of healthy men of similar age. A much greater tobacco consumption was found among the patients with cancer. Since then considerable evidence has accrued to link cigarette smoking not only with respiratory disease, but also with coronary heart disease (CHD). Changes in smoking habits may be relevant. Before 1914, tobacco consumption was mainly in the form of pipesmoking, cigars or chewing tobacco. The First World War popularised the more convenient cigarette. From 1920 to 1960 the annual consumption of manufactured cigarettes in the United States rose from 750 to 3,900 per adult. An additional factor may also

be that 93 per cent of male cigarette smokers inhale compared with 47 per cent of pipesmokers and 29 per cent of cigar smokers.

The evidence linking cigarette smoking to CHD is based on epidemiological, pathological, clinical and experimental studies. In an early study of smoking and longevity, Pearl (1939)[98] reported data from the Family History Records of Johns Hopkins Hospital. There were 2,094 white male non-smokers, 2,814 moderate smokers and 1,905 heavy smokers. Tobacco smoking was statistically associated with impairment of life duration and the degree of impairment increased directly with the amount of smoking. The differences disappeared about 70 years of age, and this Pearl attributed to a residual effect of the increased mortality of earlier years in the groups affected by the agent.

Hammond (1962)[44] undertook a prospective study of the effects of cigarette smoking. He enrolled 181,783 men between the ages of 50 and 69 and followed them for 44 months. During the time of study there were 7,316 deaths among habitual cigarette smokers. Only 4,651 of these deaths would have been expected if their death rates had matched those of non-smokers. CHD accounted for 52.1 per cent of the excess deaths and carcinoma of lung for 13.5 per cent. The CHD death rate was 77.0 per cent higher among smokers and increased progressively with the number of cigarettes smoked. In a further investigation Hammond (1969)[46] studied 447,186 men born between 1868 and 1927 and still alive on July 1st, 1960. During a five year period 39,178 died. The

estimated mean length of life remaining at age 35 was 42.4 years for men who never smoked regularly, 37.8 years for those smoking 1-9 cigarettes daily, 37.1 years for those smoking 10-19 cigarettes daily and fell to 34.7 years for those smoking more than 40 cigarettes daily. Hammond (1968)[45] also suggested that it is possible that the death rate of light smokers is apparently increased and that of heavy smokers apparently lower because of factors such as ill health which tend to reduce the number of cigarettes smoked.

Prospective clinical studies involving large populations examined at intervals have been undertaken in several centres such as Framingham and Albany in the United States. Doyle, Dawber, Kannel, Kinch and Kahn (1964)[27] reviewed the findings with regard to the relationship of smoking habits to total mortality and the incidence of new manifestations of CHD in 2,282 middle aged men surveyed for 10 years in Framingham, Mass., and 1,838 middle aged men surveyed for 8 years in Albany, N.Y. In men smoking more than 20 cigarettes daily the risk of myocardial infarction was 3 times greater than in non-smokers, former smokers, cigar and pipesmokers. No definite relationship was found between smoking and angina pectoris when this symptoms was the sole manifestation of CHD. They noted that the risk from smoking with respect to myocardial infarction remained even when blood pressure and cholesterol levels were standardised. It was also suggested that acute circulatory effects occurred due to rapid absorption of nicotine from the bronchopulmonary tree. The

results of this study differed from those of Hammond (1969)[46] in that the risk of developing myocardial infarction reverted to normal in those who gave up smoking - a finding which led Doyle to emphasise his belief in that "smoking has an acute effect on the incidence of myocardial infarction rather than necessarily contributing on a long term basis to the development of atheroma and then CHD". This interpretation has been challenged by many pathologists (vide infra). Doyle (1970)[26] subsequently stated that of the known precursors of CHD the identification of heavy cigarette smoking as a factor significantly related to myocardial infarction and sudden death was the single most important contribution that had been made through long term studies of degenerative heart disease. Gordon and Kannel (1971)[41] provided further evidence of harmful effects of cigarette smoking. Their study was again based on the Framingham population. Starting in 1948 a cohort of 5,209 residents was examined biennially. In the first 14 years, 120 died of CHD before reaching 65 years of age. Two-thirds of these deaths occurred outside hospital, the majority suddenly, within one hour of the onset of the terminal event. One half of all persons dying suddenly had no prior clinical heart disease. All of the men who died and who were apparently free of stigmata of cardiovascular disease were cigarette smokers. Spain (1969)[117] studied the pathological findings in relation to age and smoking habits in 102 men dying suddenly of a first clinical episode of CHD. The average age at death was 16 years less in heavy smokers than in non-smokers.

Of the non-smokers 21 deaths were due to CHD and the mean age was 63 years whereas 43 deaths due to CHD occurred in those smoking more than 20 cigarettes daily with mean age of death of this group being 47 years.

Epidemiological evidence thus tends to link CHD with cigarette smoking. There may be both an acute and a chronic relationship. Pathological studies have been undertaken to show that cigarette smoking may be related to atherosclerosis.

ROLE OF CIGARETTE SMOKING IN ATHEROSCLEROSIS

Cigarette smoking may lead to increased atheroma of the coronary arteries. Auerbach, Hammond and Garfinkel (1965)[6] reported on 1,509 males who had had autopsies and whose relatives had been visited to obtain details of their smoking habits during life. Their study was concerned with the 1,372 men who died of causes other than CHD. Of these men, 126 had never smoked, 893 smoked during their last illness and 353 were former cigarette, pipe or cigar smokers. Atherosclerosis of the coronary arteries was described by the size of plaques and degree of ulceration and was divided into slight, moderate and advanced. Within the age groups of less than 45 years and 45-59 years the proportion of men with a moderate to advanced degree of atherosclerosis was considerably greater among smokers than among non-smokers. Furthermore, within each age group, the degree of atherosclerosis increased consistently with the amount of cigarette smoking. Auerbach, Hammond, Garfinkel and Kirman (1971)[6] also measured the thickness of myocardial arteriolar

walls in relation to smoking and age. One aspect of the study was based on autopsy findings, the others being experimental investigations in beagle dogs. In man the thickness of the arteriolar walls was on average greater in smokers than in non-smokers and increased with age. The thickness of the arteriolar wall also increased with the number of cigarettes smoked each day and was less among cigar and pipesmokers than among cigarette smokers. In experimental studies beagle dogs daily inhaled cigarette smoke through tracheostomies while control dogs were not so exposed. The arteriolar walls became thicker in smoking dogs than in non-smoking and thicker in dogs smoking non-filter cigarettes than those smoking filter tipped cigarettes. Avtandilov, Kolenova and Ponomarenko (1965)[7] studied 400 preparations of longitudinally resected aortas and coronary arteries and made a planimetric assessment and classification of the degree of morphological manifestations of atherosclerosis. The material was divided into smokers (180) and a control group of non-smokers (220). There was a significant difference between the extent of the atherosclerosis in the intima of smokers as compared to non-smokers. In young and middle aged smokers the area of atherosclerotic lesions was 2 to 3 times greater than in controls. Sackett (1967)[111] reported on autopsies of 1,019 patients dying in a cancer hospital. These patients had been extensively questioned before death with regard to cigarette smoking and to alcohol consumption. There was a positive association between cigarette smoking and aortic atheroma. The severity of the atheroma was not related to alcohol consumption.

An important series of studies on the association of cigarette smoking and atherosclerosis has been reported by Strong and his associates (Strong, Richards, McGill, Eggen and McMurry 1969[119]; Strong and Eggen 1970[120]). Autopsies were done on 747 men between 20-64 years of age. Aortic and coronary artery lesions were evaluated both by individual dissection and by radiographs. Schedules (which had been evaluated in living subjects) were used to estimate cigarette smoking habits of the deceased men. Atherosclerotic involvement of both the aorta and coronary arteries was greatest in heavy smokers and least in non-smokers. Occupation, physical activity and educational level achieved did not account for the observed differences in the extent of the lesions.

The precise manner in which cigarette smoking promotes atherosclerotic vascular disease remains uncertain. It has been suggested that prolonged cigarette smoking may accelerate atherosclerosis through an action on fat metabolism. This theory is supported by the pathological findings of increased atheroma in smokers. There is also evidence to suggest that cigarette smoking have have an acute effect, for example, by the triggering of a fatal dysrhythmia. Theories have also been advanced that nicotine may act directly or possibly through catecholamines on cardiac metabolism and also on arterial walls.

PHYSIOLOGICAL EFFECTS OF SMOKING

Armitage and Milton (1964)[4] devised a method for introducing tobacco smoke containing known amounts of nicotine into the lungs of unanaesthetised cats. By extrapolation of the results to man

they suggested that the amount of nicotine entering the lungs of a smoker who inhales is likely to be in the range of 50-150 ug of nicotine per puff - corresponding to a dosage of 1-2 ug/kg bodyweight. It is probable that the smoking of a cigarette produces enough nicotine intake to cause a release of catecholamines from the adrenal gland in some, if not all, smokers - especially in those who inhale deeply. Less deep inhalers are more likely to have effects produced by sympathetic ganglia stimulation, release of catecholamines from the adrenal gland, chemoreceptor stimulation or even possibly by direct stimulation of the vasomotor centre. Westfall and Anderson (1967)[133] studied catecholamine storage and metabolism following cigarette smoking or nicotine administration in experimental animals. Urine samples were obtained from rats studied in metabolism cages after the administration of various dosages of nicotine (0.1 to 1.0 mg/kg). These produced a significant increase in the urinary excretion of adrenaline and metadrenaline. They concluded that release of adrenaline from the adrenal medulla was a more important cause of pharmacological reactions to smoking than noradrenaline release. Larson and Silvette (1964)[75] reviewed central nervous system function with relation to tobacco alkaloids. In general, small doses of nicotine were found to have a stimulatory action on the CNS whereas with large doses depression followed the stimulation. Doses of nicotine that will produce CNS depression or paralysis are, however, never found as a consequence of smoking. Cross circulation experiments have shown that nicotine may stimulate the cardio-inhibitor centre

directly although this direct central mechanism is less sensitive than the carotid body reflex mechanism. Minimally effective doses of nicotine appear to cause tachycardia reflexly through stimulation of chemoreceptors in the carotid and aortic bodies while larger doses also stimulate sympathetic ganglia and the adrenal medulla. Oral (1968)[97] commented that the difficulties of pharmacological investigation of nicotine are numerous and include the problem of tolerance developing in man through long continued usage of the drug, the great variability in response to similar dosage shown by normal subjects, the route of administration of nicotine affecting the results and the fact that findings in animal experiments are not necessarily applicable to man. The major action of nicotine would appear to consist of a primary transient stimulation and a secondary (probably rare) depression of all sympathetic and parasympathetic ganglia. It is due to a direct action on the ganglia cells. The action of nicotine includes the stimulation of the adrenal medulla. A low concentration can thus produce all the known effects of sympathetic stimulation on the heart and blood vessels. An increase in basal metabolic rate, free fatty acids and blood sugar has also been reported. The vasoconstrictive effect of nicotine on peripheral arterioles and arteries is due to initial stimulation of sympathetic ganglia cells with consequent discharge of impulses along post ganglionic fibres. In normal man nicotine tends to increase coronary flow or at least does not decrease it (Bargerson 1957).[9] In contrast, in a study of patients with CHD, Regan, Hellems and Bing (1960)[104] demonstrated static or decreasing coronary blood flow during cigarette smoking while blood pressure and cardiac output rose.

CIGARETTE SMOKING AND FAT METABOLISM

Food lipids are mainly triglycerides. They are hydrolysed by pancreatic lipase and then resynthesised in the intestinal mucosa and enter the blood stream as chylomicrons. These are gradually taken up by the liver. After hydrolysis to glycerol and fatty acids these acids are then re-esterified, combined with protein and re-enter the circulation as lipoproteins which then pass to adipose tissue. Incoming lipoproteins are hydrolysed by a lipoprotein lipase, resynthesised and stored. When fat depots are mobilised the stored triglycerides are again hydrolysed and the fatty acids are associated with albumin, forming water soluble albumin bound non-esterified fatty acids (free fatty acids, FFA).

Dietary carbohydrates are first taken up by the liver. Some are deposited as glycogen and some form fatty acids which are then incorporated into very low density lipoproteins (VLDL) and circulated in plasma. These are then assimilated by adipose cells by a similar mechanism as for chylomicron triglycerides.

There are 2 main processes involved in the uptake of plasma triglyceride by adipose tissue.

1. Lipoprotein lipase.
2. Fatty acid incorporation into adipose tissue.

The enzyme lipoprotein lipase, when activated under suitable conditions, is responsible for lipolysis of plasma lipoproteins. When considering fatty acid incorporation, Carlson, Walldius and Olsson (1973)[20] found evidence of a defect in fatty acid

uptake by adipose tissue of patients with raised triglyceride values. Using needle biopsy specimens of adipose tissue incubated with labelled FFA the rate of incorporation of the labelled FFA into adipose tissue was measured and found to be low in subjects with raised triglyceride values.

The process can be described schematically as follows (after Carlson 1975).[17]

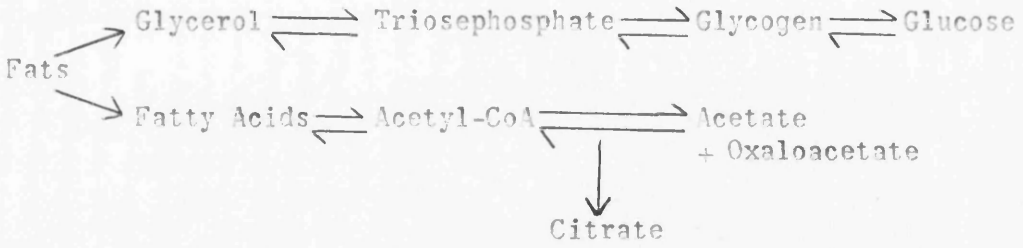
	BLOOD		FAT CELL	
Fat	Triglyceride Chylomicron Triglyceride	Fatty Acids	Fatty Acids	
Dietary	VLDL		+ Glycerophosphate	Triglyceride

Carbohydrate → Glucose

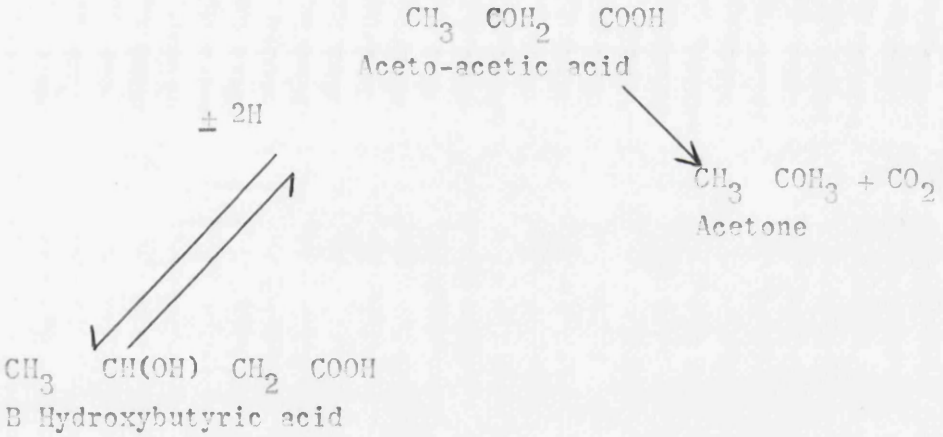
Fatty Acids — Triglyceride VLDL

LIVER CELL

Fatty acids are normally completely oxidised to carbon dioxide and water. It is likely that fatty acid chains are split into 2-carbon units consisting of some form of aceto-acetate. The entry of acetate into the citric acid cycle depends on the presence of ATP co-enzyme A and in their presence acetate is converted into acetyl-CoA. This can then react with oxaloacetate and enter the citric acid cycle.



In the liver FFA metabolism can result in the accumulation of other compounds. These are aceto-acetate together with β hydroxybutyrate and small amounts of acetone. The parent substance is aceto-acetate. Under the influences of B hydroxybutyrate dehydrogenase and NAD, aceto-acetate and β hydroxybutyrate are freely interconvertible. Acetone arises from aceto-acetate.



Traces of ketone bodies (aceto-acetate, B hydroxybutyrate and acetone) can be detected in normal blood. They increase when there is increased fat metabolism. Ketone body production is associated with non-availability or under utilisation of carbohydrate. Whether ketone bodies are regarded as direct or as secondary products of fat metabolism it has been shown that the ability of the liver

to oxidise fatty acids is limited. The liver cannot oxidise aceto-acetate although it can readily produce it. The extra-hepatic tissues can and do metabolise aceto-acetate and B hydroxybutyrate. Even when the liver is producing significant quantities of ketone bodies these may pass into the circulation and undergo oxidation in the extra hepatic tissues so that no actual ketosis develops until the rate of formation of aceto-acetate and other ketone bodies in the liver outstrips that at which they can be oxidised by other extra-hepatic tissues.

Free fatty acids may have considerable importance because of their close relationship to ventricular dysrhythmias (vide infra) and also because of the positive relationship between FFA concentration and myocardial oxygen consumption (Wahlqvist, Kaijser, Lassers and Carlson 1973)[129]. Free fatty acids appear to be taken up by the liver at a rate directly related to their plasma concentration ie an increased rate of mobilisation of FFA from adipose tissue increases hepatic fatty acid uptake. Once taken up by the liver, several metabolic procedures may occur.

(a) oxidation to carbon dioxide or ketone bodies (as described above).

(b) incorporation into VLDL triglyceride

(c) storage as liver triglyceride

Thus increased FFA mobilisation could lead to increased ketone body production and possibly to increased VLDL or even fatty liver.

Spahn and Voss (1969) in discussing the regulatory mechanisms and clinical importance of FFA stated that they represent the most important energy source of the organism in the post-absorptive phase and in conditions of increased energy requirements. Reitsma (1967) also commented that the energy supply of the organism is largely derived from FFA and glucose. During prolonged fasting FFA are the predominant fuel, glucose being mainly used to meet oxidative demands just after eating. The levels of blood sugar and FFA depend on the metabolism of adipose tissue, muscle and liver with the turnover rate of albumin bound FFA being around 30 per cent per minute. In situations where glucose utilisation is not possible, FFA levels rise rapidly while after glucose ingestion FFA levels fall. This is probably associated with the action of insulin enhancing the entry of glucose into adipose cells. This leads to the formation of alpha-glycerophosphate which is used for the esterification of FFA to form triglyceride. As a result, the output of FFA falls and remains low while glucose is available. During fasting the FFA content of plasma rises because of lipolysis. An elevated plasma FFA inhibits the utilisation of glucose by muscle which is reflected in a decrease of insulin sensitivity.

Jenkins (1967a) discussed the role of FFA and blood glucose homeostasis in diseases involving altered lipid metabolism. He suggested that raised plasma FFA and ketone body levels caused increased insulin release and could account for the raised insulin levels reported in some obese diabetic patients. This may be relevant to the glucose/fatty acid cycle theory developed by

Randle, Garland, Hales and Newsholme (1963)[103] which proposes:

(a) when blood glucose falls, FFA are liberated from adipose tissue and inhibit glucose uptake by peripheral tissues such as muscle, and

(b) the resulting rise in blood sugar in turn diminishes FFA release from adipose tissue.

Madison, Mebane, Ungar and Lochner (1964)[86] showed that infusions of ketone bodies increased insulin secretion and lowered levels of plasma FFA and glucose. This finding led them to propose that the production of ketone bodies from FFA stimulated insulin release and so limited FFA production. Insulin is believed to diminish FFA output by adipose tissue in two ways; firstly, by promoting glucose entry into adipose tissue cells and secondly, by a direct inhibitory action on lipase. Ketone bodies also inhibit FFA release by direct action on lipase. Jenkins (1967b)[55] suggested on the basis of the above work that differences in the regulation of plasma FFA, ketone bodies and glucose could be of importance in degenerative diseases of which CHD is one. It is possible that increased FFA and ketone body levels could lead to a rise in blood sugar and initially to pancreatic stimulation with perhaps eventually a state of relative pancreatic exhaustion. FFA and ketones also tend to be stimulators of gluconeogenesis and hepatic glucose output while impairing glucose utilisation in peripheral tissues. These factors all might favour increased lipid synthesis and subsequent deposition in the arterial wall.

Felts, Crofford and Park (1964) showed in experimental studies in the dog that ketone bodies could have two effects. Infusion of ketone bodies into the intact fasting dog produced prompt hypoglycaemia. They suggested that this effect is a consequence of a reduction in hepatic glucose output due to increased insulin secretion. Conversely when hyperglycaemia is established by a constant infusion of glucose, infusion of ketone bodies produces further hyperglycaemia. They proposed that this is due to an inhibition of peripheral glucose utilisation. Their reasons were as follows. Elevation of blood glucose is the optimal physiological stimulus to the pancreatic β cells and raises insulin secretion to a level where no further response can be obtained from ketone bodies. The combination of hyperglycaemia and elevated insulin levels establishes a high rate of peripheral glucose uptake. Under such conditions the inhibitory effect of ketone bodies on peripheral glucose uptake becomes manifest by a rise in blood glucose or at least a reduction of the normal tendency to fall.

Free fatty acids, ketone bodies and insulin therefore appear to be closely related in metabolic reactions. Cigarette smoking causes an increase in FFA and it has been suggested that differences in response to cigarette smoking occur between subjects with and without clinical manifestations of CHD or peripheral vascular disease (PVD). Kershbaum, Bellet, Caplan and Feinberg (1962) studied the effects of cigarette smoking in subjects who had previously had a myocardial infarction. There were 17 male patients whose myocardial infarction had occurred from three months to 12 years previously -

a considerable difference in time. Two filter cigarettes were smoked over a 10 minute period and venous blood was sampled before and 10, 20, 30 and 40 minutes after finishing the second cigarette. There was a rise of FFA which usually persisted for the 40 minutes of the study. In their clinically normal group there was a mean rise of 292 ueq/l (24.6 per cent) compared with a higher value of 856 ueq/l (65 per cent) for those subjects who had previously sustained a myocardial infarction. It was noted that the pre-smoking levels of FFA varied considerably and that there was also a variable response to smoking possible due to individual differences in the catecholamine releasing effects of nicotine. The authors suggested that the increased rise in FFA in those subjects who had had a myocardial infarction might possibly be due to increased release of adrenaline and noradrenaline from the adrenal medulla and sympathetic post-ganglionic fibres and that the myocardial infarction group might have a hyperactive adrenal/sympathetic system. However, the wide variation in time following myocardial infarction make their results difficult to evaluate fully. Kershbaum, Osada, Scriabine, Bellet and Pappajohn (1967)[62] also investigated the effect of nicotine on the mobilisation of FFA from rat adipose tissue in order to determine whether or not nicotine had any direct adiopokinetic effect contributing to the release of FFA. Epididymal fat pats were removed from decapitated rats. These were incubated and either adrenaline or nicotine was added to the control medium. There was no increase in FFA release with nicotine as compared to control values but an increase of about

100 per cent occurred with adrenaline. In further experiments, rats were given intraperitoneal injections of saline, nicotine or adrenaline two hours before sacrifice. Fat pats were then excised and incubated. FFA release was markedly increased after nicotine or adrenaline but not after saline. In a perfused dog-limb study nicotine bitartrate was given (0.5-2.5 mg/kg) intra-arterially and samples were then taken from the femoral vein at frequent intervals. There was no increase in FFA levels. Overall these results support the hypothesis that the mobilisation of FFA by nicotine and tobacco smoking is the result of their stimulation of the sympathetic nervous system and increased catecholamine secretion.

A comparative study of the effects of cigarette, cigar and pipesmoking was reported by Kershbaum (1968)[58]. Following their usual smoking habits subjects showed greater FFA mobilisation with cigarettes than with pipe or cigars. This effect was reversed when cigars were inhaled and cigarettes were not. In dogs, with the level of inhalation held constant, there was no difference in FFA or triglyceride response to cigarette, cigar or pipesmoking. Similarly the urinary catecholamine output was comparable. In man, urinary catecholamine levels were found to be increased after heavy cigarette smoking (Kershbaum, Bellet, Jiminez and Feinberg 1966)[60] when compared to control values and this effect appeared to be due to inhalation and the effect this had on nicotine absorption. It should be noted that these subjects smoked up to 20 cigarettes in order to produce this effect. They found that levels of both

adrenaline and noradrenaline in the urine increased following smoking.

It is possible that catecholamine release after nicotine affects factors other than FFA. Nestel (1964)[92] studied plasma triglyceride and FFA changes in response to noradrenaline in man. There were 17 men with CHD and eight controls. Noradrenaline was given intravenously in a dose of 0.2 ug/kg/minute for 15 minutes. A highly significant relationship was found between plasma triglyceride concentrations and the absolute and percentile increments in FFA after 15 minutes of noradrenaline infusion. At any given plasma triglyceride level the FFA response was greater in the control subject than in the patient with CHD. The inhibitory effect of nicotinic acid (200 mg) on noradrenaline induced mobilisation of FFA was also studied. In 15 subjects the fall in plasma FFA after nicotinic acid was found to be significantly related to the basal level of FFA. Nestel concluded that sympathetic nervous activity in its action of determining the magnitude of the FFA flux is a major factor in the regulation of the fasting plasma triglyceride concentration in man. Rutenberg, Schwartz and Soloff (1968)[109] studied noradrenaline and heparin induced changes in plasma FFA and compared patients with CHD to normal (but in fact much younger) controls. Noradrenaline (0.2 ug/kg/minute) and heparin 100 mg intravenously were used to determine whether their respective lipolytic activities would reveal changes in plasma FFA that might differ in such subjects. FFA increases after noradrenaline occurred more slowly and to a

significantly lesser degree in the CHD group. Although the pattern change in FFA was similar in both groups and reflected lipolysis of adipose tissue there was substantially less rise in the percentage of palmitoleic acid than the normal both in terms of absolute amounts and as a percentage of total FFA. Sixty minutes after heparin there was a significantly greater amount of stearic acid still present in the plasma of the CHD group than in the normal controls. Heparin was also given 5 minutes after stopping the noradrenaline infusion. Then minutes later there was a significantly greater level of the two saturated acids, myristic and palmitic in the CHD group. The rate of decline of total and individual FFA was much faster after the higher levelsof FFA which were reached by the combined effects of noradrenaline and heparin.

Kingsbury and Jarrett (1967)[65] studied the effects of adrenaline and smoking in patients with PVD. They measured glucose, immunoreactive insulin (IRI) and FFA in 36 male subjects with clinically severe PVD. The effects of smoking two non-tipped cigarettes over 20 minutes and adrenaline 0.01 ul of 1:10,000 solution/kg bodyweight was compared. Under basal conditions, the FFA response to both adrenaline and smoking was within normal limits. Adrenaline consistently produced a rise in blood sugar whereas smoking either had no effect or lowered the fasting glucose level. In 5 patients, however, smoking caused a rise in IRI which could not be accounted for by change of blood sugar. Kingsbury and Jarrett suggested that this response to smoking might occur in people who are hypersecretors of insulin. They commented that the effects of

smoking have up to now been regarded as due to release of catecholamines but that other effects should be considered. In some subjects, there may be an enhanced insulin response perhaps due to the action of nicotine as a vagal stimulant. It should be noted that Kingsbury and Jarrett reported that adrenaline caused a rise in IRI. This findings is in contra-distinction to that reported by Porte, Graber, Kuzuyat and Williams (1966)[102] who also investigated the effects of adrenaline on IRI levels in man. During the infusion of 6 ug/minute of adrenaline in normal subjects there was marked hyperglycaemia but IRI remained at basal levels until the infusion was stopped. This inhibition of an expected increase in IRI could be maintained for up to 7 hours by the continued infusion of adrenaline. Their data were considered to be consistent with an inhibitory effect of adrenaline upon pancreatic insulin release.

The overall impression is that there is a significant rise in FFA following cigarette smoking. Whether or not this is excessive in subjects who have CHD has been postulated but not yet proven. There does seem to be some evidence than an increased response occurs in those subjects who have had a myocardial infarction as an expression of CHD. Whether this is a permanent or transient phenomenon has not yet been established. It has also usually been assumed that the reason for the increased FFA following cigarette smoking is a consequence of sympathetic and adrenal medullary stimulation with release of catecholamines causing lipolysis of adipose tissue. It has also been suggested that insulin release may have a part to play

in some patients. Little consideration has been given to the fate of these increased FFA especially with regard to the level of ketone body production and perhaps also to the rate of metabolism of glycerol released during lipolysis. Ketone body production would seem to be especially relevant in regard to regulation of blood glucose, insulin and possibly even FFA levels in a type of feedback control.

The importance of an increase in FFA after cigarette smoking could lie in two directions. These would include the long term increased development of atheroma leading to an increased incidence of myocardial infarction. This might be reflected in higher lipid levels in smokers as compared to non-smokers. There could also be acute effects of cigarette smoking in causing release of catecholamines and rise in FFA. It has been suggested that such events are likely to be associated with, and perhaps responsible for, the development of major cardiac dysrhythmias.

METABOLIC ROLE OF GLYCEROL

Adipose tissue, once regarded as metabolically inert, is now recognised to contain a wide range of enzymes catalysing many synthetic and degradative processes. Moreover, these enzymatic activities are high, indeed comparable in many instances to levels of enzyme activity found in the liver when considered in relation to the protoplasmic content of adipose tissue.

The only method for breakdown of triglycerides in adipose tissue that has so far been demonstrated is the hydrolytic cleavage of the ester bonds - i.e. lipolysis. The rate of release of free glycerol and FFA from glyceride complexes is not impaired when intact tissues are incubated anaerobically or in the presence of fluoride suggesting that glyceride degradation does not require energy (Vaughan 1962)[127]. It is gradually believed that lipoprotein lipase is not involved in the mobilisation of FFA and glycerol from adipose tissue. This enzyme functions in the transfer of lipid into adipose tissue from circulating lipoproteins. Lipolysis, on the other hand, is affected by means of hormone sensitive lipase activity. In homogenates, the level of this enzyme correlates directly with observed changes in glycerol production in intact tissues. The rate of production of glycerol is increased when intact adipose tissue is incubated with one of the fat mobilising hormones which include adrenaline, noradrenaline, adrenocorticotrophic hormone (ACTH) and thyroid stimulating hormone (TSH).

TRIGLYCERIDE SYNTHESIS

Under physiological conditions, glycerol seems to have little part to play in this process. Some studies, however, using high concentrations of substrate have suggested alternate paths for the formation of ∝ glycerophosphate. The pathway of triglyceride synthesis has been investigated by measuring the incorporation of isotopically labelled fatty acids or ∝ glycerophosphate into glyceride in homogenates of rat epididymal adipose tissue. The following pathways have been suggested (Vaughan and Steinberg 1965)[127].

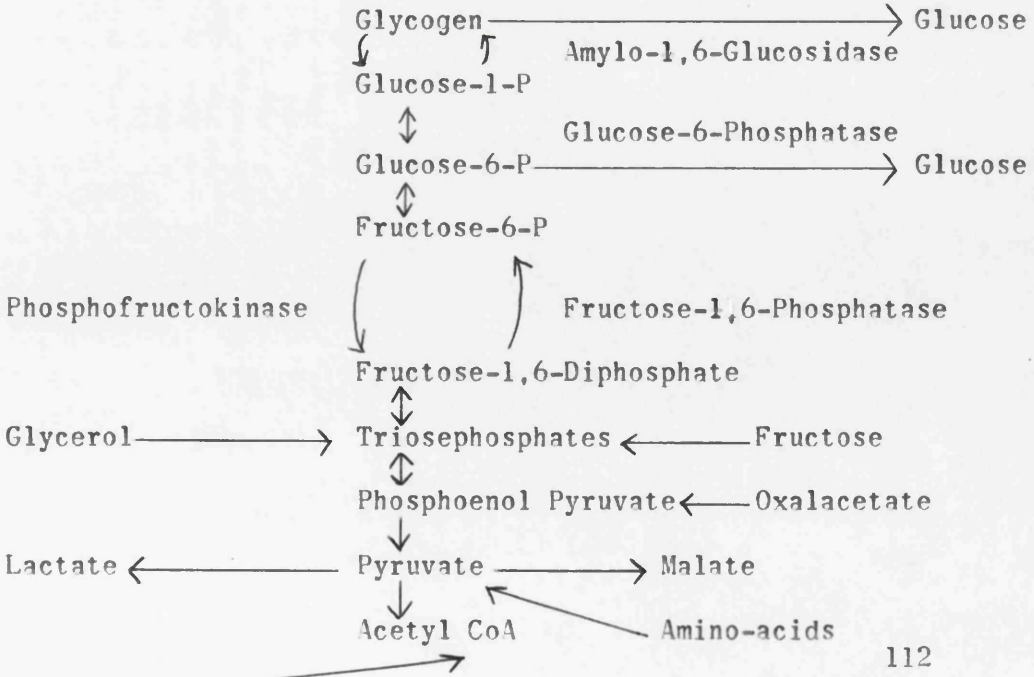
1. Fatty Acid + CoA + ATP ⟶ Fatty Acyl CoA + AMP + PP
2. 2 Fatty Acyl CoA + GP ⟶ Phosphatidic Acid + 2 CoA
3. Phosphatidic Acid ⟶ Diglyceride + Phosphate
4. Diglyceride + Fatty Acyl CoA ⟶ Triglyceride + CoA

Glycerol cannot replace ∝ glycerophosphate in reaction (2) although it has been possible to demonstrate some ester formation when millimolar amounts of glycerol are added. Glycogen, glucose-1-P or glucose do not support esterification in the absence of ∝ glycerophosphate and glycerol does not replace ∝ glycerophosphate in this system. It was found, however, that about 0.1% of a tracer amount of 1.3C^{14} glycerol was incorporated into glyceride glycerol. In other studies, synthesis of ∝ glycerophosphate was found from glycerol by transphorylation in a system containing phenylphosphate, glycerol and magnesium chloride. Although these studies demonstrate

alternate pathways of ∝ glycerophosphate generation high concentrations of substrate were used and their physiological significance is believed to be dubious.

GLYCEROL RELEASE FOLLOWING LIPOLYSIS

The glycerol which is released from adipose tissue during lipolysis is a major endogenous precursor of newly formed glucose. After phosphorylation in the liver over 70% of the triosephosphate is converted to glucose and glycogen, 5% approximately is oxidised to carbon dioxide and around 3% is accounted for by lactate production. The rate of use of glycerol reflects the efficiency of the gluconeogenic pathways. Glycerol may have an anti-ketotic effect in vivo by supplying carbohydrate necessary for the oxidation of acetate by the Krebs cycle. It can apparently supply those carbohydrate intermediates necessary for the normal functioning of the Krebs cycle.



112

Metabolic pathways of glycerol (Senior and Loridan 1968)

Carlson and Oro (1963)[19] studied the relationship between the concentration of plasma FFA and glycerol. They found a significant correlation between the fasting levels of FFA and glycerol. Noradrenaline administration increased and glucose administration decreased both FFA and glycerol. They considered that their data showed a close relationship under various conditions between FFA and glycerol in keeping with the hypothesis that they were both derived from lipolysis of triglyceride in adipose tissue.

Tibbling (1969)[122] suggested that studies of glycerol turnover might be a better index of fat metabolism than measurements of FFA concentration. He used two methods to study glycerol kinetics. These were (1) the continuous infusion method and (2) the rate of disappearance of glycerol after administration as a single intravenous injection. Comparable results were obtained with each method. His main investigations were done in euthyroid and hyperthyroid subjects. The mean fractitonal turnover rate of glycerol in hyperthyroid subjects was increased by 15% compared to euthyroid subjects, suggesting a slight effect of hyperthyroidism on the fraction of glycerol eliminated. The mean blood glycerol, however, was increased by 210% and the mean glycerol turnover rate by 230%, findings consistent with a marked increase of fat metabolism in hyperthyroidism.

There is some variation in the disappearance rates of glycerol reported in human studies. Senior and Loridan (1968)[112]

administered glycerol intravenously as a 10% solution in isotonic saline in a dose of 120 mm per square metre of body surface area. Nine adults and 13 children were studied. The mean glycerol T½ was 39.3 ± 4.3 minutes. The values could be expressed as a single exponential. Tibbling (1969)[123] studied glycerol disappearance after a single injection of intravenous glycerol in four euthyroid men. The meausred half times were 17.2, 17.3, 18.4 and 23.4 minutes. These corresponded well with measurements made in the same individuals using the continuous infusion method. Pelkonen and his associates (1967)[100] found that T½ of glycerol ranged between 10.7 - 12.3 minutes. Their results were calculated on the basis of very rapid mixing in the glycerol distribution phase with dynamic equilibrium occurring around ten minutes after injection. On the other hand Tibbling felt that equilibrium was not reached until twenty minutes or so after injection. He felt that differences in disappearance rates could be related to the measurement of different sections of the curve. He did agree that the data could be represented by a straight line semi-logarithmic plot, implying a first order reaction.

Overall it seemed possible that a study of glycerol kinetics would be of interest in the context of coronary heart disease and cigarette smoking. Previous work has mainly dealt with FFA mobilisation after cigarette smoking rather than on the fate of glycerol released during lipolysis.

EFFECT OF CIGARETTE SMOKING ON BLOOD LIPID LEVELS

Most of the work done in man has been in relation to cholesterol levels. Howell (1970)[52] in a study of 2483 males aged 40-51 years showed no significant variation in either cholesterol or beta-lipoprotein levels (crudely measured) in heavy smokers, non-smokers and ex-smokers. In the Carterton Study (Evans, Prior and Cooke 1969)[32] the serum cholesterol values of a sample of town dwelling New Zealanders of European stock were presented. Smoking habits were graded on a 9 category scale. No relationship was found between smoking and cholesterol levels. Kontinnen (1962)[67] measured the total serum cholesterol and cholesterol content of the alpha- and beta-lipoprotein fractions and serum phospholipids in 314 healthy young men aged from 18-25 years. He found no difference relating to smoking habits in any of the variables measured. Leren (1970),[80] in his report on the Oslo diet-heart study also found no relationship between smoking and cholesterol levels. Wahl and Schlettler (1968)[128] found no recognisable differences in the cholesterol levels of smokers and non-smokers: nor was there any evidence that triglyceride levels were influenced by cigarette smoking. Indeed, Kontinnen and Rajasalni (1963)[68] found that after cigarette smoking there was a decrease in the normal post-prandial rise in serum triglycerides. This was attributed (on no good evidence) to sympathetic nervous system stimulation. Contrary results were reported by Karvonen, Orma, Keys, Fidanza and Brozek (1959)[57] who found higher than average serum cholesterol levels in all ages (other than 50-59) for those who smoked compared to those who did not. For example, smokers aged 40-49 years had a mean

cholesterol level of 244 mg/100 ml compared to the non-smokers value of 220 mg/100 ml.

The effect of cigarette smoking on blood lipid levels was investigated during a study of the prevalence of lipoprotein abnormalities in the West of Scotland (Lorimer, Cox, Greaves, Jubb, Hawthorne, Morgan and Lawrie 1974)[83]. The sample consisted of apparently healthy men, either self-employed or on the staff of professional or industrial groups. The survey comprised 4477 men (response rate 7790). There were 2346 (52.3%) non-manual and 2131 (47.7%) manual workers. Before attending the screening unit, each subject was asked to complete a questionnaire containing standardised questions on history of chest pain (Rose and Blackburn 1968a)[106] and smoking habits. Height, weight, blood pressure (Rose and Blackburn 1968b)[107] a 6 lead electrocardiogram (3 standard and 3 unipolar limb leads) and chest radiograph were recorded.

Venous blood, 10 ml, was drawn from subjects who had been asked to fast for at least 9 hours before sampling. Plasma was separated and, when necessary, stored at $4^{o}C$. Plasma total cholesterol and triglycerides were measured by auto-analyser techniques (Annan and Isherwood 1969[3]; Kessler and Lederer 1966)[63]. Plasma lipoprotein electrophoresis was carried out on each sample using albuminated barbitone buffer (Lees and Hatch 1963)[78]. Subjects who had probable post-prandial hypertriglyceridaemia on the basis of chylomicrons detected on the electrophoretogram were reinstructed

about the need for fasting and sampling was repeated.

The following arbitrary definitions were used in the classification of subjects studied. Cigarette smoking - 5 or more cigarettes daily. Obesity - more than 10% above predicted ideal weight. Hypertension - systolic blood pressure of more than 170 mmHg and/or diastolic pressure more than 100 mmHg. Electrocardiographic abnormalities were those summarised as categories I and II in the Minnesota code and related mainly to Q waves, ST depression and T wave inversion. A history of possible angina was obtained from the questionnaire. With these criteria, the classification was as follows.

TABLE I HEALTH SURVEY IN WEST OF SCOTLAND MEN

CLASSIFICATION	PERCENTAGE
NORMAL	29.5
CIGARETTE SMOKING	59.0
OBESE	28.0
HYPERTENSIVE	7.4
ABNORMAL ELECTROCARDIOGRAM	4.9
POSSIBLE ANGINA	5.0

One thousand three hundred and eight subjects had no apparent abnormality and were regarded as the "normal" group. A similar number (again 1308) of subjects smoked cigarettes but had no other abnormality and were regarded as the "normal apart from cigarette smoking" group. This is of course not the total

number smoking since many with an abnormality such as hypertension or obesity were also cigarette smokers.

The comparison of fasting lipid values in the two groups (Tables 2A and B) showed that in general plasma cholesterol values tend to be slightly lower in the subjects smoking more than 5 cigarettes daily. There is no tendency for them to have hypercholesterolaemia. Fasting triglyceride values are overall slightly higher in the subjects who smoke. Both groups are within normal weight range so that this is not an effect of obesity.

In an experimental study (Kershbaum, Bellet and Khorsandian 1965)[61] the effect of nicotine on cholesterol levels was investigated. Twenty dogs were given intramuscular nicotine (0.5 mg/kg) daily for 4 weeks then 1.0 mg/kg daily for 2 weeks. This dose is 500-1000 times more than that absorbed by a heavy smoker over a comparable time interval. The mean serum cholesterol level rose from 144 $\pm$ 11.8 mg/100 ml to 216 $\pm$ 15.3 mg/100 ml ($P < .05$). The rise in cholesterol was maintained until the administration of nicotine was stopped. The relevance of this study to human cigarette smoking is at best uncertain. Overall it was felt that these changes could be due to a nicotine induced rise in FFA. Triglyceride levels were not measured in this study.

Overall, therefore, it would appear that there is no convincing evidence in man to suggest that cigarette smoking leads to a consistent rise in cholesterol levels. The same may be true for triglyceride levels though this is less well established. Pathological evidence already cited does suggest an increase in

TABLE 2A

FASTING PLASMA CHOLESTEROL VALUES IN "NORMAL" SUBJECTS AND IN THOSE WITH SMOKING AS THE SINGLE ABNORMALITY

	"NORMAL"			CIGARETTE SMOKERS		
AGE	NO.	MEAN	S.D.	NO.	MEAN	S.D.
< 39	238	216	39	192	218	40
40-44	362	227	38	322	225	38
45-49	279	233	39	333	225	40
50-54	247	234	38	266	227	39
55-59	160	232	48	183	222	37
60	22	246	40	12	240	51
TOTAL	1308	229	39	1308	224	39

TABLE 2B

FASTING PLASMA TRIGLYCERIDE VALUES IN "NORMAL" SUBJECTS AND IN THOSE WITH SMOKING AS THE SINGLE ABNORMALITY

	"NORMAL"			CIGARETTE SMOKERS		
AGE	NO.	MEAN	S.D.	NO.	MEAN	S.D.
< 39	239	102	77	191	113	65
40-44	360	111	63	322	123	79
45-49	279	109	51	333	120	66
50-54	246	105	48	266	123	95
55-59	159	104	44	179	110	71
60	22	93	30	12	97	33
TOTAL	1305	107	59	1303	118	76

aortic and coronary atheroma in cigarette smokers and it may be that more subtle metabolic changes are induced by cigarette smoking which are subsequently reflected in premature or accelerated atherosclerosis. In addition, transient metabolic abnormalities could be important in the triggering of a CHD event.

RELATIONSHIP BETWEEN MYOCARDIAL INFARCTION, FREE FATTY ACIDS, CATECHOLAMINES AND DYSRHYTHMIAS

Using the concentrations of urinary free adrenaline and noradrenaline as indices of adrenergic drive a picture has been built up of the relationship between clinical syndromes, haemodynamic changes and adrenergic activity after myocardial infarction. Those with the most severe clinical illness appear to have the highest catecholamine excretion values. Insofar as dysrhythmias are concerned it is difficult to decide on the relationship. McDonald, Baker, Bray, McDonald and Restieaux (1969)[85] recorded higher plasma noradrenaline values in post infarction patients than occurred in normal controls. Compared with patients who had no complications, patients with atrial dysrhythmias or early ventricular dysrhythmias had higher plasma noradrenaline levels although this was not found in patients whose ventricular dysrhythmias occurred later in their illness. Further aspects of a possible relationship were discussed in the Lancet (1969)[76]. It was stated that a correlation between catecholamines and presence or absence of dysrhythmias can be only tentatively accepted since plasma catecholamine concentration is only one component of

neurohumoral sympathetic drive. Furthermore, increased catecholamine excretion is more obvious in seriously ill patients who often have circulatory failure so that they may have a more severe infarction and be more liable to rhythm disturbances. There may be both biological advantages and disadvantages to adrenergic drive. Thus in many patients with circulatory failure, maintenance of myocardial contractility and blood pressure by catecholamines is necessary for survival although on the other hand catecholamines have been shown to be capable of producing myocardial necrosis. After experimental coronary occlusion, catecholamines are discharged from the ischaemic myocardium although it is unlikely that this accounts for much of the rise reported after infarction. A generalised leak of noradrenaline from sympathetic nerve endings and adrenal medullary discharge of adrenaline seems more likely. There has also been considerable discussion as to the relevance of increased FFA to the ischaemic heart and to the production of dysrhythmias (Kurien and Oliver 1966[71]; Oliver, Kurien and Greenwood 1968[95]; Kurien, Yates and Oliver 1969; Kurien and Oliver 1970[73]); Opie, Thomas, Owen, Norris, Holland and Van Noorden 1971[96]; Nelson 1970[91]; Gupta, Jewitt, Young, Hartog and Opie 1969[42]).

Oliver, Kurien and Greenwood (1968[95]), studied the relationship between serum FFA, dysrhythmias and death following acute myocardial infarction. Serial FFA measurements were made in 200 patients during the first 48 hours after acute myocardial infarction. Those with a striking elevation ($>$1200 ueq/l) had an increased prevalence of both serious dysrhythmias and disorders of conduction.

Both early and late deaths were more frequent among these patients compared with those who had smaller increases. Similar relationships did not occur insofar as enzymes such as alanine and aspartate transferase were concerned. These authors suggested that the relationship between FFA levels and dysrhythmias could result from increased catecholamine activity or it could be due directly to an increase in myocardial oxygen consumption caused by the utilisation of FFA as the major energy substrate. Either or both mechanisms would intensify myocardial hypoxia in an already ischaemic myocardium. Kurien, Yates and Oliver (1969)[73] discussed heparin, FFA and dysrhythmias during experimental myocardial infarction. Elevation of plasma FFA was achieved through the activation of lipoprotein-lipase by heparin and was associated with the development of serious ventricular dysrhythmias in dogs with acute myocardial infarction. Pretreatment with protamine sulphate prevented elevation of FFA and this reduction in FFA levels was associated with a decrease in the frequency of ventricular dysrhythmias. They suggested that their findings were compatible with the hypothesis that high levels of FFA could by themselves cause dysrhythmias and that this relationship was not necessarily due to other influences responsible for the mobilisation of FFA. They commented that FFA are increased after acute myocardial infarction and that they had previously shown that high levels of FFA are associated with an increased incidence of dysrhythmic deaths. While this rise in FFA is probably due to increased catecholamines and perhaps also linked to cortisol (Logan and Murdoch 1966)[88] these

factors may not be directly responsible for the development of dysrhythmias. Increased concentrations of FFA may themselves damage cells (Hoak, Connor, Warner 1968)[50]. Kurien et al found that there was a time lag of a few minutes between maximum observed FFA level and peak incidence of ectopic beats which could indicate that absolute plasma FFA levels are not immediately responsible for the induction of dysrhythmias. An intracellular defect (perhaps a cell membrane action) could lead to altered permeability. The ability of the cells to deal with unbound FFA by esterification or oxidation may be exceeded when extracellular concentrations are high and electrical activity could be impaired by FFA cellular accumulation.

It should be pointed out that, although highly suggestive, the evidence linking raised concentrations of FFA with dysrhythmias has not yet been conclusively established under all conditions. Thus Opie, Thomas, Owen, Norris, Holland and Van Noorden (1971)[96] discussed the failure of high concentrations of circulating FFA to provoke dysrhythmias following experimental myocardial infarction. The possible dysrhythmogenic effect of high plasma FFA levels was studied in 30 dogs with myocardial infarction induced by occlusion of the anterior descending coronary artery. In no animal was it clear that high plasma FFA concentrations by themselves provoked or exaggerated ectopic activity. They believed that their results argued against a major role for high plasma FFA concentrations in the genesis of severe dysrhythmias following myocardial infarction. Opie and his group required adrenaline infusion in addition to marked FFA elevation before they could find any evidence of ectopic

activity after the combination of intralipid and heparin. There were, however, differences between their study and that of Kurien, Yates and Oliver. Thus Opie studied greyhounds and used open chested techniques with ligation of anterior descending coronary artery whereas Oliver's group studied mongrels and used balloon occlusion in close chested animals with occlusion of the circumflex coronary artery.

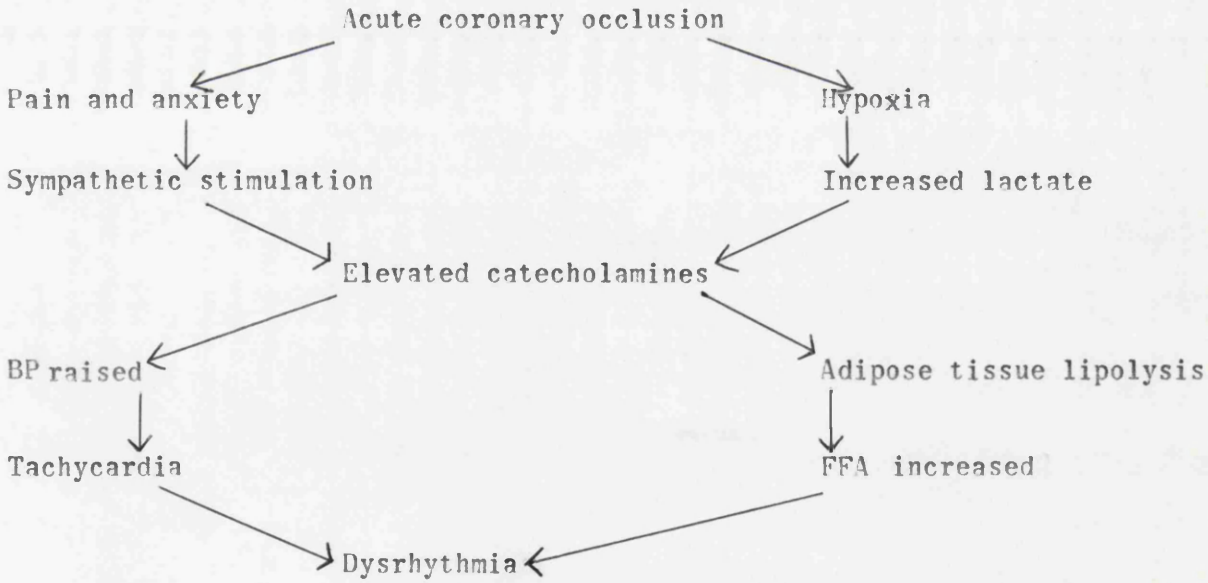
Nelson (1970)[91] studied the effects of heparin on FFA and catecholamines and incidence of dysrhythmias following acute myocardial infarction in man. Heparin, given in a therapeutic dose, produced a significant rise in FFA within 10 minutes of injection but did not increase the incidence of dysrhythmias, nor did heparin have any significant effect on plasma catecholamine levels. Gupta, Jewitt, Young, Hartog and Opie (1969)[42] also investigated raised FFA levels and their significance in patients with acute myocardial infarction. Plasma FFA and urinary catecholamine excretion rates were measured in 35 patients with acute myocardial infarction. High initial plasma FFA values ($>$1000 ueq/l) were associated with increased evidence of serious cardiac dysrhythmias. The excretion of urinary free adrenaline correlated with fasting plasma FFA levels although this relationship was not found with urinary free noradrenaline. They believed that their study had not established a direct role for high FFA values in the production of cardiac dysrhythmias following infarction. It may be that increased FFA uptake by the ischaemic myocardium is a factor in the production of such dysrhythmias or it may

equally well be that elevated FFA values reflect some other abnormality such as increased catecholamine secretion which could be the more direct cause of the dysrhythmia.

Thus we have a situation in which epidemiological studies suggest that cigarette smokers have a higher incidence of sudden death than non-smokers. There is work to suggest that elevated FFA levels following myocardial infarction are associated with an increased incidence of dysrhythmias and the cigarette smoking causes a rise in FFA. It may be that these are linked. Bellet, DeGuzman, Kostis, Roman and Fleischmann (1972)[12] studied the effect of inhalation of cigarette smoke on ventricular fibrillation threshold in normal dogs and dogs with an acute myocardial infarction. The electrical impulses were delivered through the chest wall in one group whereas in another a gated series of impulses was delivered directly to the heart through previously implanted epicardial electrodes. A decrease in ventricular threshold that averaged 30-40% of control values was observed in normal dogs as well as in those with experimental myocardial infarction. This effect of inhalation of cigarette smoke was evident 30 minutes after smoking, became maximum at about 45 minutes and lasted about 90-120 minutes. In dogs with acute myocardial infarction the ventricular fibrillation threshold was lower than in normal dogs and was decreased further after inhalation of cigarette smoke. As the authors state, these findings are of interest in view of the increased incidence of sudden death among coronary patients who are heavy cigarette smokers.

CHAPTER TWO - AIMS OF THE STUDY

From the work cited in the introduction there is evidence to suggest that those who smoke cigarettes are at increased risk from CHD and its complications. This relates both to sudden death and also to increased likelihood of cardiac dysrhythmias in those subjects with elevated FFA levels. These levels seem also to be associated with elevated catecholamine values which in themselves could be harmful. There is controversy over the direct role of FFA as a major factor in producing dysrhythmias. Insofar as myocardial infarction is concerned the following scheme has been postulated (Kurien and Oliver 1970).[72]



Cigarette smoking has been shown to produce rises in FFA values probably secondary to catecholamine release from sympathetic nerve

endings, adrenal medulla or chromaffin cells in the heart. It has also been suggested that subjects with known previous myocardial infarction respond to cigarette smoking with a greater than normal increase in FFA values. This could be harmful to the myocardium, conducive to the development of tachydysrhythmias and a predisposing factor to sudden death as well as perhaps leading to increased coronary artery atherogenesis.

It has also been reported that there are certain subjects with peripheral vascular disease (PVD) who respond to cigarette smoking with insulin hypersecretion - a factor which could also be associated with increased atherogenesis. This, however, is a fact not in keeping with experimental evidence showing that adrenaline lowers insulin levels but which is more consistent with the suggestion that ketone bodies produced by the liver in the metabolism of FFA, might act as stimulators of insulin secretion.

Most work has been concerned with the measurement of FFA levels in a variety of situations based on the theory of increased lipolysis of adipose tissue. Little has been reported on possible differences in metabolism of the FFA and whether or not subjects with differing clinical conditions and different lipoprotein types respond in similar or different ways to the stimulus of cigarette smoking. Glycerol as well as FFA is produced by lipolysis and changes in glycerol metabolism following cigarette smoking have not been well documented.

It was, therefore, decided to undertake a series of investigations into the effect of smoking both normal nicotine content and low

nicotine content cigarettes in normal subjects, subjects 3 weeks and one year following myocardial infarction, subjects with angina and subjects with clinical PVD to contrast and compare the effects on glucose, FFA, ketone bodies, cholesterol, triglyceride and insulin levels. A further series of investigations was also done into the pattern of FFA released after smoking in normal and CHD subjects to study the role of individual FFA. Finally the effect of cigarette smoking on glycerol kinetics was carried out in subjects with PVD and in normal controls.

CHAPTER THREE - SUBJECTS AND METHODS

In all studies subjects were fasted overnight and were kept in bed in the semi-supine position on the morning of the study. An 18F gauge plastic cannula (Argyle Medicut) was introduced into a median antecubital vein following subcutaneous local anaesthetic (29% lignocaine). The cannula was attached to a 3 way tap and kept patent by the slow (1 ml/min) infusion of normal saline. The cannula was left in situ for 30 minutes before samples were obtained, since initial investigations had shown that there was a transitory rise in FFA following insertion of the cannula but that a steady state was reached in 30 minutes. Blood samples were obtained as required from the 3 way tap obviating the need for further venepuncture. Samples were taken into lithium heparin containers for measurement of plasma FFA, cholesterol, triglyceride and insulin; into fluoride for measurement of blood glucose and into ice-cold perchloric acid for measurement of ketone bodies.

When assessing the effects of cigarette smoking, the following protocol was followed.

1. Insertion of cannula.
2. After 30 minutes base line sample obtained.
3. 2 cigarettes smoked with 10 minutes being taken for each cigarette, and with a 5 minutes interval between cigarettes.
4. Blood samples obtained 15, 30, 45 and 60 minutes after smoking the second cigarette.

Each subject had the procedure explained to him and gave consent to the investigation.

METHODS

1. BLOOD GLUCOSE

Blood glucose was measured by the enzymatic determination of true glucose using a Boehringer Mannheim kit, based on the method of Keston (1956).[64]

This involves the use of the following reagents.

Solution 1 Buffer enzyme solution. 0.1M phosphate buffer pH 7.0; 40 ug peroxidase; 250 ug glucose oxidase/ml. Made up to 150 ml in distilled water.

Solution 2 Chromogen (6.6 mg O-dianisidene-HCL/ml.

Solution 3 Glucose standard solution (91 ug glucose/ml).

Solution 4 This was made up by mixing 100 parts of solution 1 with 1 part of solution 2, stirring vigorously. This stable in a dark bottle for 1 day at room temperature and was brought up to 20^{o}C before use.

Perchloric acid. 2.85 ml of 70% perchloric acid was made up to 100 ml with redistilled water. 0.10 ml blood from a fluoride container and 1.00 ml of perchloric acid were pipetted into a 10 ml centrifuge tube. This was centrifuged and 0.2 ml of the deproteinised supernatant pipetted into a dry test tube. Blanks and standard solutions were prepared and the optical densities of the solutions read at 480 nm using a glass cuvette with a 1 cm light path in a Unicam SP600 spectrophotometer.

Results were obtained as mg glucose/100 ml blood.

2. NON-ESTERIFIED FATTY ACIDS (free fatty acids; FFA)

In the majority of studies the method used was that described by Dole (1956).[25] Unless otherwise stated this was the method used for FFA measurement. In some subsequent studies, additional methods were used and were those described by Trout, Estes and Friedberg (1960)[124] and that provided by Boehringer Mannheim based on the method of Duncombe (1964).[28]

DOLE'S METHOD

1 ml of heparinised plasma was extracted with 5 ml of a mixture of isopropanol 40: heptane 10: in sulphuric acid 1. To this mixture was added 2 ml heptane and 3 ml water. This separated into 2 phases. 2 ml of the top layer was removed and to it was added 1 ml of Nile Blue Indicator (0.02%) and titrated with approximately 0.018N sodium hydroxide. Standards of known concentrations of palmitic acid were also titrated. The results of the unknown solution were obtained and expressed as microequivalents per litre (ueq/l).

METHOD OF TROUT, ESTES AND FRIEDBERG

2 ml of heparinised plasma were extracted with 10 ml of the extraction mixture as used by Dole. 6 ml heptane and 4 ml distilled water is added to the mixture and shaken for at least 2 minutes. A 5 ml aliquot of the heptane layer is pipetted into a beaker and 5 ml of 0.05% aqueous sulphuric acid added. The mixture is shaken vigorously and then centrifuged. 3 ml of the heptane layer is titrated with approximately 0.018 N sodium hydroxide using 1 ml of Nile Blue A

solution (0.02N) as indicator. Standard solutions are similarly titrated and results calculated as in Dole's method.

BOEHRINGER MANNHEIM METHOD

5 ml of chloroform are pipetted into 3 test-tubes, blank, standard and test. 0.2 ml of a standard (0.5 meq/l non-esterified fatty acids) are added to the standard tube and 0.2 ml of serum is added to the test. To all 3 tubes is added 1 ml of a solution of 0.27M copper nitrate in 0.45M triethanolamine buffer pH 7.8. After shaking vigorously for 10 minutes, the aqueous blue green layer is drawn off by a water vacuum pump. 2 ml portions of each chloroform layer are pipetted into test tubes and 0.2 ml of 9 mm diethyldithiocarbamate solution added. After mixing optical densities were read at 440 nm on a Unicam SP600 spectrophotometer and the results calculated as meq/l.

3. ACETO-ACETATE AND β HYDROXYBUTYRATE

Aceto-acetate was measured using a Boehringer Mannheim kit, based on the method of Bergmeyer and Brenot (1965).[13]

THEORY OF REACTION

Aceto-acetate reacts with reduced nicotinamide dinucleotide (NADH) in the presence of β hydroxydehydrogenase to yield hydroxybutyrate and nicotinamide dinucleotide (NAD). The rate of consumption of NADH is proportional to the amount of aceto-acetate and can be determined on the basis of its absorption at 340 nm.

$$\text{Aceto-acetate} + \text{NADH} + H^+ \underset{\text{(}\beta\text{ OH dehydrogenase)}}{\rightleftharpoons} \text{D-(-)-3-OH Butyrate} + NAD^+$$

The estimation of β hydroxybutyrate utilises the same reaction but in reverse.

$$\beta\text{ OH Butyrate} + NAD^+ \underset{\text{(}\beta\text{ OH dehydrogenase)}}{\rightleftharpoons} \text{Aceto-acetate} + \text{NADH}$$

METHOD FOR ACETO-ACETATE

3 ml of freshly drawn venous blood was added to 3 ml of 0.7M perchloric acid in an ice-bath. This precipitated the blood proteins and the mixture was then centrifuged at 3,000 rpm for 10 minutes.

0.5 ml of IM tripotassium phosphate solution was added to 2.5 ml of supernatent. This was mixed and left in an ice-bath for 30 minutes and then spun at 2,500 rpm for 5 minutes. The mixture was then allowed to reach room temperature. Into a 1 cm light path glass cuvette water-jacketted at 25°C was put 2 ml of filtrate (buffered to pH 7.5 with tripotassium phosphate) and 0.05 ml of NADH solution. These were mixed and the reaction observed using a Unicam SP800 spectrophotometer at wavelength 340 nm. Once the reaction stopped 0.01 ml suspension of lactic dehydrogenase and malic dehydrogenase (LDH/MDH) was added. Optical density was remeasured 10 minutes after the reaction stopped (some conditions as before). 0.02 ml of hydroybutyrate dehydrogenase suspension added and the optical density read after 22, 24, 26 and 28 minutes.

Aceto-acetate was calculated by subtracting the final (28 min) reading from the reading obtained after the LDH/MDH reaction finished.

3.9 x difference in reading = mg aceto-acetate/100 ml blood.

METHOD FOR B HYDROXYBUTYRATE

5 ml of freshly drawn venous blood was added to 5 ml of IM perchloric acid in an icebath. After protein precipitation the mixture was spun at 3,000 rpm for 10 minutes. To 5.0 ml of supernatant was added 0.3 ml of 0.025 M NAD solution and 0.65 ml of 3M potassium hydroxide/IM potassium carbonate solution. This was mixed and stood in an ice-bath for 30 minutes after which the precipitated potassium perchlorate was filtered off. The filtrate (buffered to pH 9.5 with potassium hydroxide/potassium carbonate solution) was allowed to rise to a temperature of 25°C. 3.5 ml of filtrate was put into a 1 cm light path water-jacketted cuvette at 25°C. The optical density was read and then 0.02 ml of β hydroxybutyrate dehydrogenase suspension was added. This was mixed and allowed to stand for 30 minutes. Again using the Unicam SP800 spectrophotometer at wavelength 340 nm the optical density was measured at 5 minute intervals from 30 minutes after beginning of the reaction until reaction completed.

β hydroxybutyrate was calculated by subtracting reading 1 from reading 2.

6.52 x difference in reading = mg β hydroxybutyrate/100 ml blood.

4. TRIGLYCERIDE

Triglyceride levels were measured by the method of Van Handel and Zilversmit (1957).[126]

2 ml of chloroform was added to 4 g of ducil. After shaking 1 ml of plasma was added. After further shaking 18 ml of chloroform were added. This was stoppered and shaken intermittently for 10 minutes and then filtrated through Whatman No 43 filter paper. 1 ml of filtrate was pipetted into a test tube. Using a stock solution of 1 g tripalmitin in 100 ml chloroform, standards were set up containing 50, 100 and 150 ug/ml as well as a blank solution of 1 ml chloroform.

All were blown down to dryness under nitrogen in a water bath at 30°C. 0.5 ml of 0.1 N alcoholic potassium hydroxide was added. This was mixed, stoppered and placed in a water bath at 70°C for 20 minutes. 0.5 ml of 0.4 N sulphuric acid was added followed by 0.1 ml of 0.05 M sodium periodate. This was mixed and allowed to stand for 10 minutes. 0.2 ml of 20% sodium sulphite solution. 0.5 ml of 0.4 M sodium periodate. 0.5 ml of 0.4 M sodium periodate. This was mixed and allowed to stand for 10 minutes. 0.2 ml of 20% sodium sulphite solution and 7.5 ml of chromatropic acid (990 ml of concentrated sulphuric acid to 450 ml of water) was added plus 60 ml of 3.36 g of 4.5 dihydroxy .2.7 naphthalene disulphonic acid disodium salt made up to 60 ml with water). This was mixed, stoppered and placed in a boiling water bath for 30 minutes. After cooling slightly 1 ml of 4.6% thiourea solution was added. All samples were mixed and read at 570 nm on a Unicam SP600 spectrophotometer.

5. CHOLESTEROL

Cholesterol levels were measured by the method of Pearson, Stern and McGavack (1953).[99]

To 0.2 ml of serum was added 2 ml of colour stabiliser (7.5% toluene P-sulphonic acid was made up in 25% acetic acid in acetic anhydride vv). This was allowed to stand for 10 minutes in a water bath at 37°C. A standard cholesterol solution of 200 mg/100 ml was similarly prepared. 2 ml of acid reagent was added (15% sulphuric acid in diluent of 25% acetic acid in acetic anhydride vv). Optical density was read in a Unicam SP600 spectrophotometer at 625 nm and cholesterol value calculated.

6. GLYCEROL METHOD

Glycerol levels were measured using the Boehringer Mannheim kit, based on the method of Eggstein (1966).[29]

Solution 1 0.1M triethanolamine buffer pH 7.6; 4 mM magnesium sulphate.

Solution 2 6 mM NADH; 33 mM ATP, 11 mM phosphoenol pyruvate.

Solution 3 2 mg LDH/ml. 1 ml pyruvate kinase/ml.

Solution 4 2 mg glycerol kinase ml.

Contents of bottle 1 are dissolved in 150 ml distilled water.

Contents of bottle 2 are dissolved in 2 ml distilled water.

Solutions 3 and 4 are suspensions.

(a) FREE GLYCEROL

0.5 ml of fresh, non-haemolysed serum was added to 2.5 ml solution 1, 0.1 ml solution 2 and 0.02 ml solution 3.

This was mixed and allowed to stand in a cuvette for 10 minutes at room temperature. Optical density was read on a Unicam SP800 spectrophotometer at 340 nm. Using a constriction pipette, 0.02 ml of suspension 4 was added and a further reading made in 10 minutes.

R1-R2 x 9.3 = mg glycerol/100 ml.

(b) TOTAL GLYCEROL

To obtain this value the serum was saponified using alcoholic potassium hydroxide.

0.2 ml of serum was added to 0.5 ml alcoholic potassium hydroxide. This was mixed and put in a water bath at 70°C for 30 minutes. After cooling 1 ml of magnesium sulphate was added. This was filtered and the method then followed as for free glycerol using 0.5 ml of filtrate in place of serum.

R1-R2 x 79 (conversion factor) = Total glycerol mg/100 ml.

(c) GLYCERIDE GLYCEROL

This is calculated by subtracting free from total glycerol. Neutral fat (triglyceride) is obtained thus

9.61 x mg glyceride glycerol/100 ml = Neutral fat mg/100 ml

7. PLASMA INSULIN

Plasma insulin was assayed by the immunoprecipitation technique of Hales and Randle (1963).[43] Standards and anti-sera were as described by Buchanan and McKiddie (1967).[15]

GAS-LIQUID CHROMATOGRAPHY (GLC) METHOD

FFA and triglyceride were extracted as follows. Five ml of serum was added to 100 ml chloroform; methanol (2:1 vv). This was extracted over 24 hours being shaken intermittently for the first 6 hours. It was then filtered through Whatman No 41 paper and the residue washed twice with 10 ml of the chloroform: methanol mixture. Fifty ml of 0.9% saline were layered on to the filtrate and left overnight. The chloroform layer was then removed and dried with sodium sulphate which was then washed with chlorofrm and the contents of the flask brought to dryness.

The extract was run on thin layer chromatography plates using silica gel G and a solvent system of hexane 90: ether 20: methanol 3: acetic acid 2. The plate was then sprayed with fluorescein solution and the separated FFA and triglyceride bands visualised. Occasionally triglyceride did not separate sufficiently from cholesterol esters. When this occurred the cholesterol ester: triglyceride band was scraped off and re-run in the solvent system benzene: chloroform 4: 1 vv.

The FFA and triglyceride bands were scraped from the plate into tubes and extracted by chloroform from the silica gel and then blown down to dryness under nitrogen. The FFA and triglycerides were then methylated with boron trifluoride 14% in methanol. For triglyceride methylation 1 ml of 14% boron trifluoride: benzene: methanol (25: 20: 55 vv) was added to the extract and placed in a boiling water bath for 30 minutes. For FFA methylation 1 ml of

14% boron trifluoride was added to the extract and then placed in a boiling water bath for 2 minutes.

After methylation 2 ml hexane and 1 ml water was added and the mixture shaken. The hexane layer was removed and evaporated to dryness. The extract was dissolved in hexane before applying to the GLC column using a Hamilton Syringe. The column consisted of 12.5% diethylene glycol succinate on 100-120 mesh Gas chrom Q and the column temperature was 190° with a nitrogen flow of 50-60 ml/minute (GLC apparatus Pye 104). The consistent fatty acid patterns were obtained from a Kent recorder.

CHAPTER FOUR

VERIFICATION OF STEADY STATE CONDITIONS

This chapter describes the investigations undertaken to confirm the presence of a steady state and to measure the effects of stimuli other than smoking on the resting levels of variables such as glucose, FFA and ketone bodies.

Study A To assess the effect of the introduction of a venous cannula and subsequent serial venous sampling on levels of glucose, FFA, ketone bodies, cholesterol, triglyceride and insulin in control subjects and subjects 3 weeks after myocardial infarction.

There were 6 subjects in the control group. All were male, age range 34-48 years, mean 42 years. Their diagnoses were mitral stenosis (3), hypertension (2) and obesity (1). The 6 subjects studied after recent infarction were all male, age range 50-59 years, mean 54 years.

The investigation was carried out as described previously in the methods section with serial venous sampling being done 15, 30, 45 and 60 minutes after obtaining a baseline sample.

The results (Table 3) show that a satisfactory steady state is achieved in terms of values for FFA and ketone bodies. Serial venous sampling done atraumatically through an indwelling cannula and tap did not alter the value of any variable. Resting values for FFA, aceto-acetate and β OH butyrate were higher in the post-infarction group than in the controls ($P<0.05$) but, as with control subjects,

did not alter with repeated sampling. As might be expected cholesterol and triglyceride values were higher in the post-infarction group but there was no difference in insulin levels.

Study B To assess the effect of a subcutaneous injection of 0.5 ml saline on levels of glucose, FFA, ketone bodies, cholesterol, triglyceride and insulin in control subjects and subjects 3 weeks after myocardial infarction.

There were 6 subjects in the control group. All were male, age range 36-56 years, mean 46 years. Their diagnoses were mitral stenosis (3), indiopathic hypertension (2) and duodenal ulcer (1). The 6 subjects studied after recent infarction were also all male, age range 44-63 years, mean 53 years.

The conditions of investigation were as described with serial venous samples being taken 15, 30, 45 and 60 minutes after the saline injection. Tables 2A and 2B show the absolute and percentage changes found.

Results indicated that the fasting baseline levels of FFA, aceto-acetate and β OH butyrate were significantly higher ($P<0.05$) in the post infarction group as compared to control subjects. Both groups showed a similar consistent slight rise in FFA and ketones following saline injection, but this did not reach significant levels. There was no tendency for the post-infarction group to have a greater response in any of the variables measured after the noxious stimulus of an injection then occurred in control subjects. This suggested that the model was satisfactory to study the effects of cigarette smoking.

Study C To assess the effect of smoking 2 low nicotine content cigarettes on levels of glucose, FFA and ketone bodies in control subjects and subjects 3 weeks after myocardial infarction.

There were 6 subjects in the control group. All were male, age range 40-54 years, mean 47 years. Their diagnoses were mitral valve disease (3), hypertension (2) and obesity (1). All were habitual cigarette smokers (10-20 daily). The 6 subjects studied 3 weeks after myocardial infarction were all male, age range 42-57 years, mean 50 years. All had been habitual smokers (approximately 20 daily) prior to infarction and had resumed smoking subsequently.

The investigation was carried out as described previously with serial venous samples being taken before and 15, 30, 45 and 60 minutes after smoking 2 low nicotine content cigarettes. Each cigarette was smoked ~~and~~ OVER a 10 minute period with a 5 minute interval between. They had a nicotine content of approximately 0.4-0.5 mg nicotine which is less than half the nicotine content of a standard size non-tipped cigarette and were supplied by the Tobacco Research Council. When ~~Such~~ low nicotine content cigarettes were smoked ~~and~~ subjects were not told of their reduced nicotine content.

The results are shown in Table 3 and show that smoking 2 low nicotine content cigarettes did not significantly increase the levels of any of the measured variables. As before the basal levels of FFA and ketone bodies were higher in the post-infarction group than in control subjects. This could perhaps represent a state of residually increased sympathetic and perhaps adrenal medullary function following and persisting after myocardial infarction. Post-infarction however

did not differ from control subjects in their response to smoking low nicotine cigarettes. This suggests that these subjects had no undue "stress" reaction to the act of cigarette smoking in terms of an enhanced pharmacological change. Such an effect might have occurred - and so obscured the responses to smoking standard cigarettes - since subjects following myocardial infarction had been counselled to avoid cigarette smoking but had not done so.

TIME	AGE	GLUCOSE (mg/100 ml) 0	15	30	45	60	F.F.A. (ueq/l) 0	15	30	45	60	ACETO-ACETATE (mg/100 ml) 0	15	30	45	β OH BUTYRATE (mg/100 ml) 0	15	30	45
CONTROL	42 +- 6	94 +- 6	95 +- 2	96 +- 3	93 +- 4	92 +- 4	738 +- 143	750 +- 138	740 +- 138	755 +- 139	748 +- 140	0.29 +- .09	0.31 +- .10	0.30 +- .08	0.29 +- .07	1.22 +- .14	1.24 +- .14	1.24 +- .14	1.24 +- .12
POST INFARCT	54 +- 4	93 +- 9	94 +- 8	92 +- 11	92 +- 9	92 +- 9	847 +- 150	852 +- 171	843 +- 161	846 +- 163	838 +- 151	0.39 +- .13	0.40 +- .15	0.41 +- .13	0.41 +- .13	1.32 +- .26	1.34 +- .29	1.33 +- .28	1.34 +- .29

	CHOLESTEROL (mg/100 ml) 0	15	30	45	60	TRIGLYCERIDE (mg/100 ml) 0	15	30	45	60	INSULIN (units/ml) 0	15	30	45	60
CONTROL	200 +- 21	202 +- 23	203 +- 23	203 +- 23	201 +- 23	107 +- 25	107 +- 25	109 +- 24	108 +- 23	105 +- 23	35 +- 12	35 +- 13	33 +- 12	33 +- 10	33 +- 11
POST INFARCT	237 +- 30	237 +- 34	236 +- 35	239 +- 32	239 +- 30	124 +- 23	127 +- 24	127 +- 24	127 +- 23	128 +- 25	31 +- 15	30 +- 17	29 +- 18	33 +- 21	33 +- 18

TABLE 3 EFFECT OF INTRODUCTION OF VENOUS CANNULA AND SERIAL VENOUS SAMPLING ON LEVELS OF GLUCOSE, F.F.A., KETONE BODIES, CHOLESTEROL, TRIGLYCERIDE AND INSULIN IN CONTROL SUBJECTS AND SUBJECTS 3 WEEKS AFTER MYOCARDIAL INFARCTION

Mean ± SD is shown

TIME	AGE	GLUCOSE (mg/100 ml)					F.F.A. (ueq/l)					ACETO-ACETATE (mg/100 ml)				β OH BUTYRATE (mg/100 ml)			
		0	15	30	45	60	0	15	30	45	60	0	15	30	45	0	15	30	45
CONTROL	46 +- 8	87 +- 8	87 +- 10	87 +- 8	87 +- 8	86 +- 9	677 +- 156	745 +- 170	761 +- 183	733 +- 167	680 +- 154	0.24 +- .03	0.28 +- .02	0.29 +- .04	0.27 +- .03	1.11 +- .12	1.20 +- .15	1.24 +- .12	1.18 +- .10
POST INFARCT	53 +- 7	95 +- 10	99 +- 8	99 +- 7	97 +- 8	98 +- 9	860 +- 109	950 +- 106	975 +- 176	966 +- 169	922 +- 135	0.40 +- .20	0.48 +- .19	0.46 +- .17	0.44 +- .17	1.39 +- .20	1.58 +- .30	1.60 +- .33	1.47 +- .20

TIME	CHOLESTEROL (mg/100 ml)					TRIGLYCERIDE (mg/100 ml)					INSULIN (units/ml)				
	0	15	30	45	60	0	15	30	45	60	0	15	30	45	60
CONTROL	235 +- 36	235 +- 31	236 +- 36	236 +- 33	236 +- 35	111 +- 19	110 +- 19	115 +- 21	114 +- 20	109 +- 18	25 +- 7	27 +- 6	24 +- 9	21 +- 8	26 +- 9
POST INFARCT	239 +- 58	270 +- 56	268 +- 57	267 +- 59	268 +- 56	203 +- 122	204 +- 124	203 +- 121	204 +- 124	203 +- 124	30 +- 10	26 +- 9	24 +- 7	24 +- 9	27 +- 8

TABLE 4A

EFFECT OF SUBCUTANEOUS INJECTION OF 0.5 ML OF SALINE ON LEVELS OF GLUCOSE, F.F.A., KETONE BODIES, CHOLESTEROL, TRIGLYCERIDE AND INSULIN IN CONTROL SUBJECTS AND IN SUBJECTS 3 WEEKS AFTER MYOCARDIAL INFARCTION

ABSOLUTE VALUES ± SD IS SHOWN

TIME	F.F.A.				ACETO-ACETATE			β OH BUTYRATE			INSULIN			
	15	30	45	60	15	30	45	15	30	45	15	30	45	60
CONTROL	9 +- 8	13 +- 9	9 +- 9	1 +- 3	18 +- 12	20 +- 12	12 +- 15	7 +- 4	12 +- 8	7 +- 8	6 +- 9	-22 +- 23	-15 +- 18	5 +- 28
POST INFARCT	11 +- 10	13 +- 15	12 +- 16	8 +- 11	23 +- 22	23 +- 30	15 +- 22	14 +- 17	16 +- 21	9 +- 17	-10 +- 12	-10 +- 12	-13 +- 13	-5 +- 11

TABLE 4B EFFECT OF SUBCUTANEOUS INJECTION OF 0.5 ML OF SALINE ON LEVELS OF GLUCOSE, F.F.A., KETONE BODIES, CHOLESTEROL, TRIGLYCERIDE AND INSULIN IN CONTROL SUBJECTS AND IN SUBJECTS 3 WEEKS AFTER MYOCARDIAL INFARCTION

PERCENTAGE CHANGE ± SD IS SHOWN

TIME	AGE	GLUCOSE (mg/100 ml) 0	15	30	45	60	F.F.A. (ueq/l) 0	15	30	45	60	ACETO-ACETATE (mg/100 ml) 0	15	30	45	β OH BUTYRATE (mg/100 ml) 0	15	30	45
CONTROL	47 +- 5	92 +- 5	91 +- 6	90 +- 8	92 +- 7	92 +- 5	682 +- 109	713 +- 110	687 +- 113	701 +- 123	701 +- 123	0.32 +- .11	0.36 +- .13	0.38 +- .13	0.37 +- .13	1.32 +- .13	1.37 +- .16	1.41 +- .21	1.39 +- .20
POST INFARCT	50 +- 5	95 +- 8	96 +- 9	98 +- 8	96 +- 7	96 +- 10	991 +- 179	1015 +- 167	1024 +- 200	1044 +- 225	992 +- 169	0.44 +- .32	0.48 +- .32	0.47 +- .33	0.48 +- .35	1.41 +- .46	1.44 +- .41	1.53 +- .44	1.47 +- .51

TABLE 5 EFFECT OF SMOKING 2 LOW NICOTINE CONTENT CIGARETTES ON LEVELS OF GLUCOSE, F.F.A. AND KETONE BODIES IN CONTROL SUBJECTS AND IN SUBJECTS 3 WEEKS AFTER MYOCARDIAL INFARCTION

Mean ± SD is shown

CHAPTER FIVE

RESPONSE TO CIGARETTE SMOKING FOLLOWING RECENT MYOCARDIAL INFARCTION AND IN SUBJECTS WITH ANGINA PECTORIS OR PERIPHERAL VASCULAR DISEASE

This chapter describes studies undertaken to investigate the metabolic responses (in terms of FFA, ketone bodies etc.) to cigarette smoking in subjects who had suffered a recent myocardial infarction, in subjects with coronary heart disease presenting as angina pectoris and subjects presenting with peripheral vascular disease (PVD) contrasted and compared to subjects with no evidence of vascular disease.

Study A To assess the effect of smoking 2 non-tipped normal nicotine content cigarettes (each containing approximately 1.4 mg nicotine) on levels of glucose, FFA, ketone bodies cholesterol, triglyceride and insulin in control subjects and subjects 3 weeks after myocardial infarction.

There were 10 subjects in the control group. All were male, age range 35-54 years, mean 45 years. Their diagnoses were mitral valve disease (4), idiopathic systemic hypertension (4), renal colic (1) and lone atrial fibrillation (1). Fourteen subjects were studied 3 weeks after myocardial infarction. All had smoked more than 20 cigarettes daily prior to infarction and at the time of study were smoking approximately 5 cigarettes daily. All subjects were male, age range 42-63 years, mean 51 years.

Conditions of investigation were as previously described, with serial venous samples being taken 25, 30, 45 and 60 minutes after smoking.

The results (Tables 5A and B, Figures 1 and 2) show that smoking 2 normal cigarettes produced a significant rise ($P<0.05$ to 0.01) in FFA levels at 15, 30 and 45 minutes in both control and post infarct group. The levels at T60 in both groups were returning towards baseline values. The FFA values were significantly higher ($P<0.01$) at T0 and at all subsequent time intervals in the post infarction group. Aceto-acetate and β OH butyrate values rose in both groups following cigarette smoking but the values in the post infarction group although higher, were not significantly so at less than the 5% level when compared with control subjects.

There was a considerable variation in FFA levels reached after smoking when individual subjects are compared. Fifteen minutes after smoking the percentage increase in FFA values in the control group ranged from 25 to 100 per cent, whereas in the post infarction group the increase ranged from 24 to 142 per cent with overall of course ~~or~~ a greater mean percentage increase in ~~their~~ this group.

It should be pointed out that although both groups had a significant rise in FFA levels the increases in the post infarction group were significantly greater than the control subjects (at T15 $P<0.05$, at T30 $P<0.01$, at T45 $P<0.01$, at T60 $P<0.01$).

In terms of insulin response to smoking there was no change in insulin levels and no differences were found between control and

and post infarction subjects. Levels of glucose remained unaltered, suggesting that the probable catecholamine release as a consequence of smoking was sufficient to alter FFA values but insufficient to affect glucose levels.

Values for cholesterol and triglyceride likewise remained constant also suggesting that, in the short term at least, cigarette smoking does not increase or affect circulating cholesterol or triglyceride levels.

Study B To assess the effect of smoking 2 non-tipped normal nicotine content cigarettes on levels of glucose, FFA, ketone bodies and insulin in subjects presenting with peripheral vascular disease (PVD) and in those presenting with angina pectoris due to CHD.

There were 9 subjects in the PVD group. All were male, age range 44-61 years, mean 54 years. All had symptoms of severe intermittent claudication with angiographically demonstrated atherosclerosis of the lower limb vessels. Done ~~Here~~ complained of exertional chest pain and all had normal electrocardiograms. It is recognised that this does not exclude underlying CHD but this was not manifest clinically. All had smoked more than 20 cigarettes daily for several years.

There were 6 subjects with angina. Again all were male, age range 39-53 years, mean 45 years. All had abnormal (ischaemic) resting electrocardiograms and had coronary artery disease demonstrated by coronary angiography. Each smoked 15-20 cigarettes daily.

The conditions and methods of investigation were as previously outlined.

The results (Tables 7A and B, Figure 3) show that there was a significant increase ($P<0.05$ to $P<0.01$) in FFA and ketone bodies in both groups. The rise in FFA, when expressed as a percentage of the initial value was significantly greater in the angina than in the PVD group. This increase in FFA value occurred in the presence of a borderline fasting FFA value that was slightly lower in the angina than in PVD subjects. There was no significant difference in the levels of ketone body release that occurred after cigarette smoking when the angina and PVD groups are compared. The overall response to cigarette smoking was similar in angina and control subjects but less, in times of FFA release, in the PVD group.

As before, smoking 2 normal cigarettes did not produce any change in levels of insulin, cholesterol and triglyceride.

Table 8 and Figure 4 indicates the incremental and total changes in FFA and ketone body values that were found. The total production of FFA and ketone bodies were obtained by subtracting basal values from those measured at T15, T30, T45 and T60 and the values were then summed (ie T15-T0 + T30-T0 etc). The production of FFA and ketone bodies was higher in the post infarction subjects than in the other groups. The lowest FFA response was found in the PVD group, a fact possibly related to the ~~habitual extent~~ continuous nature of their cigarette smoking. Figure 5 suggests that in general alterations in FFA levels are reflected by parallel changes in ketone body levels.

TIME	AGE	GLUCOSE (mg/100 ml) 0 15 30 45 60	FFA (ueq/l) 0 15 30 45 60	ACETO-ACETATE (mg/100 ml) 0 15 30 45	β OH BUTYRATE (mg/100 ml) 0 15 30 45
CONTROL	45 ± 6	96 99 99 96 98 ± 14 16 15 16 17	716 899 953 846 758 ± 110 172 182 143 123	0.25 0.35 0.41 0.34 ± .10 .11 .12 .11	1.13 1.48 1.55 1.43 ± .27 .33 .32 .33
POST INFARCT	51 ± 7	87 88 86 87 85 ± 9 10 10 10 10	869 1136 1193 1166 1043 ± 167 188 250 254 190	0.26 0.45 0.52 0.46 ± .12 .24 .24 .22	1.07 1.54 1.73 1.52 ± .14 .26 .36 .36

TIME	CHOLESTEROL (mg/100 ml) 0 15 30 45 60	TRIGLYCERIDE (mg/100 ml) 0 15 30 45 60	INSULIN (units/ml) 0 15 30 45 60
CONTROL	225 225 224 224 224 ± 48 50 50 48 49	107 108 108 107 107 ± 22 23 22 23 22	29 28 28 29 27 ± 11 10 9 10 10
POST INFARCT	250 251 252 256 250 ± 50 50 51 49 50	127 127 129 126 126 ± 51 50 54 52 52	24 24 22 25 24 ± 12 9 8 10 10

TABLE 6A EFFECT OF SMOKING 2 NON-TIPPED CIGARETTES ON LEVELS OF GLUCOSE, FFA, KETONE BODIES, CHOLESTEROL, TRIGLYCERIDE AND INSULIN IN CONTROL SUBJECTS AND IN SUBJECTS 3 WEEKS AFTER MYOCARDIAL INFARCTION

Absolute value ± SD is shown

	FFA	ACETO-ACETATE	β OH BUTYRATE
TIME	15 30 45 60	15 30 45	15 30 45
CONTROL	27 35 22 10 ± ± ± ± 18 13 14 12	36 64 43 ± ± ± 17 27 17	26 40 29 ± ± ± 16 22 17
POST INFARCT	35 43 39 27 ± ± ± ± 22 37 43 39	64 97 78 ± ± ± 42 44 52	47 65 43 ± ± ± 35 50 41

TABLE 6B EFFECT OF SMOKING 2 NON-TIPPED CIGARETTES ON LEVELS OF GLUCOSE, FFA, KETONE BODIES, CHOLESTEROL, TRIGLYCERIDE AND INSULIN IN CONTROL SUBJECTS AND IN SUBJECTS 3 WEEKS AFTER MYOCARDIAL INFARCTION

Percentage change ± SD is shown

TIME	AGE	GLUCOSE (mg/100 ml) 0 15 30 45 60	FFA (ueq/l) 0 15 30 45 60	ACETO-ACETATE (mg/100 ml) 0 15 30 45	β OH BUTYRATE (mg/100 ml) 0 15 30 45
PVD	55 ± 8	93 93 94 59 94 ± 12 ± 11 ± 13 ± 14 ± 13	773 855 891 859 803 ± 58 ± 80 ± 118 ± 111 ± 65	0.24 0.32 0.35 0.29 ± .09 ± .07 ± .10 ± .10	1.10 1.37 1.46 1.29 ± .23 ± .31 ± .43 ± .46
ANGINA	45 ± 9	95 98 94 97 94 ± 10 ± 12 ± 8 ± 11 ± 9	638 797 861 780 693 ± 114 ± 159 ± 139 ± 107 ± 105	0.28 0.41 0.41 0.32 ± .08 ± .09 ± .12 ± .09	1.32 1.74 1.76 1.57 ± .35 ± .36 ± .42 ± .32

TIME	INSULIN (units/ml) 0 15 30 45 60	CHOLESTEROL (mg/100 ml) 0 60	TRIGLYCERIDE (mg/100 ml) 0 60
PVD	21 21 19 22 22 ± 8 ± 9 ± 7 ± 11 ± 12	240 242 ± 34 ± 36	127 124 ± 45 ± 46
ANGINA	24 22 23 21 21 ± 11 ± 12 ± 13 ± 11 ± 11	262 260 ± 21 ± 23	128 125 ± 13 ± 16

TABLE 7A EFFECT OF SMOKING 2 NON-TIPPED CIGARETTES ON LEVELS OF GLUCOSE, FFA, KETONE BODIES AND INSULIN IN SUBJECTS WITH PVD AND SUBJECTS WITH ANGINA

Absolute value ± SD is shown

	FFA				ACETO-ACETATE			β OH BUTYRATE		
	15	30	45	60	15	30	45	15	30	45
PVD	10 ± 7	14 ± 15	11 ± 15	4 ± 10	48 ± 25	65 ± 51	29 ± 42	31 ± 32	38 ± 38	21 ± 34
ANGINA	25 ± 8	36 ± 13	27 ± 16	10 ± 7	57 ± 25	57 ± 14	20 ± 13	37 ± 20	37 ± 4	17 ± 8

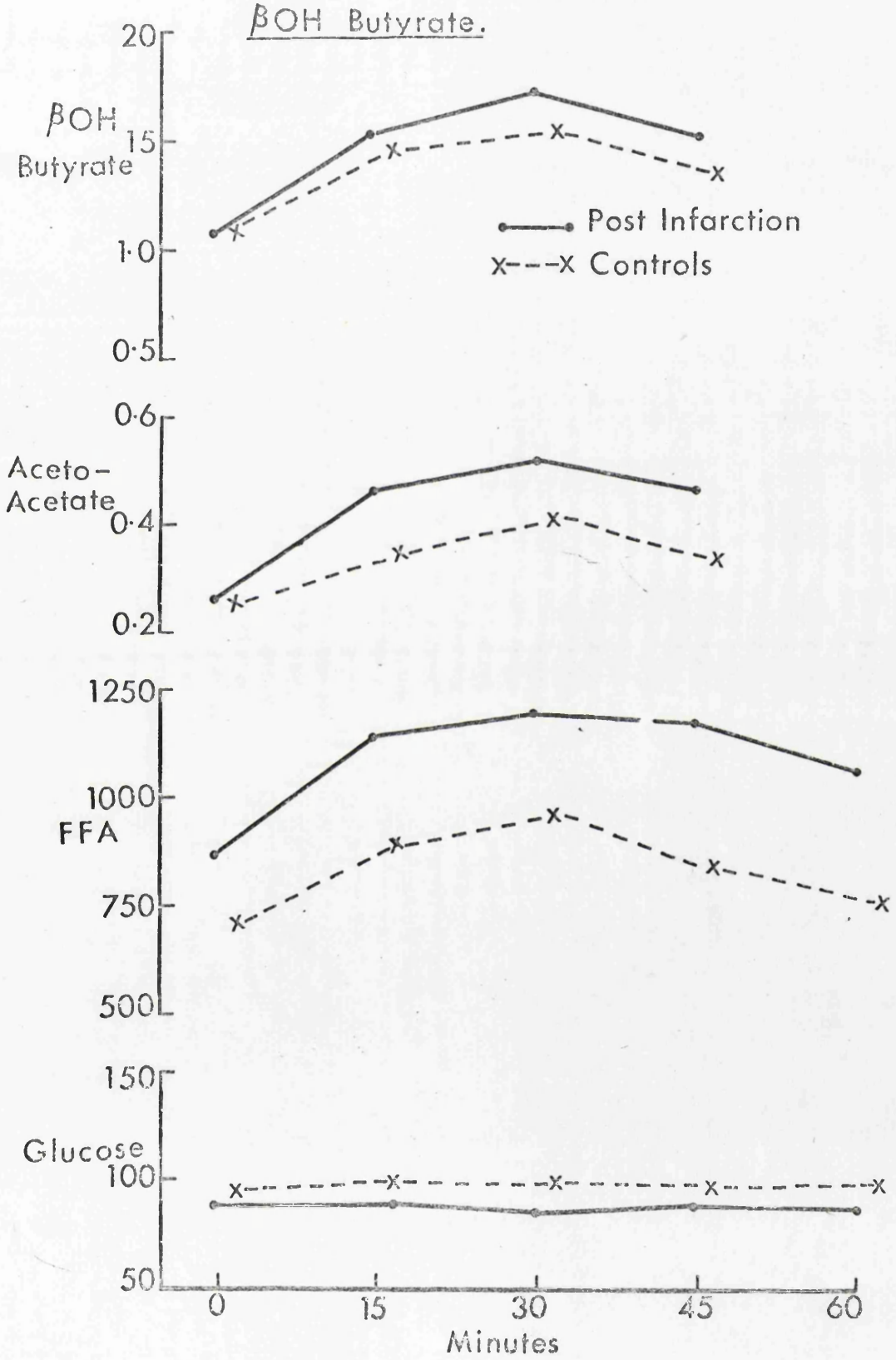
TABLE 7B EFFECT OF SMOKING 2 NON-TIPPED CIGARETTES ON LEVELS OF GLUCOSE, FFA, KETONE BODIES AND INSULIN IN SUBJECTS WITH PVD AND SUBJECTS WITH ANGINA

Percentage change ± SD is shown

GROUP / TIME		FFA INCREASE ueq/l 15	30	45	60	TOTAL	ACETO-ACETATE INCREASE mg/100 ml 15	35	40	TOTAL	β OH BUTYRATE INCREASE mg/100 ml 15	30	45	TOTAL
CONTROL	10	188 +- 134	240 +- 123	143 +- 98	66 +- 69	639	0.09 +- .06	0.16 +- .08	0.10 +- .08	.35	.27 +- .16	.42 +- .23	.30 +- .25	.99
MYOCARDIAL INFARCT	12	253 +- 156	327 +- 191	273 +- 189	141 +- 151	994	0.12 +- .06	0.19 +- .07	0.16 +- .11	.47	0.37 +- .18	0.51 +- .20	0.34 +- .22	1.22
PVD	9	82 +- 52	123 +- 107	97 +- 104	39 +- 50	341	0.10 +- .07	0.13 +- .08	0.08 +- .08	.31	0.30 +- .31	0.41 +- .41	0.23 +- .39	.94

TABLE 8 INCREMENTS OF FFA AND KETONE BODIES FOUND AFTER SMOKING 2 NORMAL CIGARETTES IN CONTROL SUBJECTS, THOSE FOLLOWING A RECENT MYOCARDIAL INFARCTION AND THOSE WITH PVD

Effect of Smoking 2 normal cigarettes on levels of Glucose, FFA, Aceto-Acetate and βOH Butyrate.



Percentage change in levels of FFA, Aceto-Acetate and βOH Butyrate after smoking 2 normal cigarettes in control and Post Infarct Subjects.

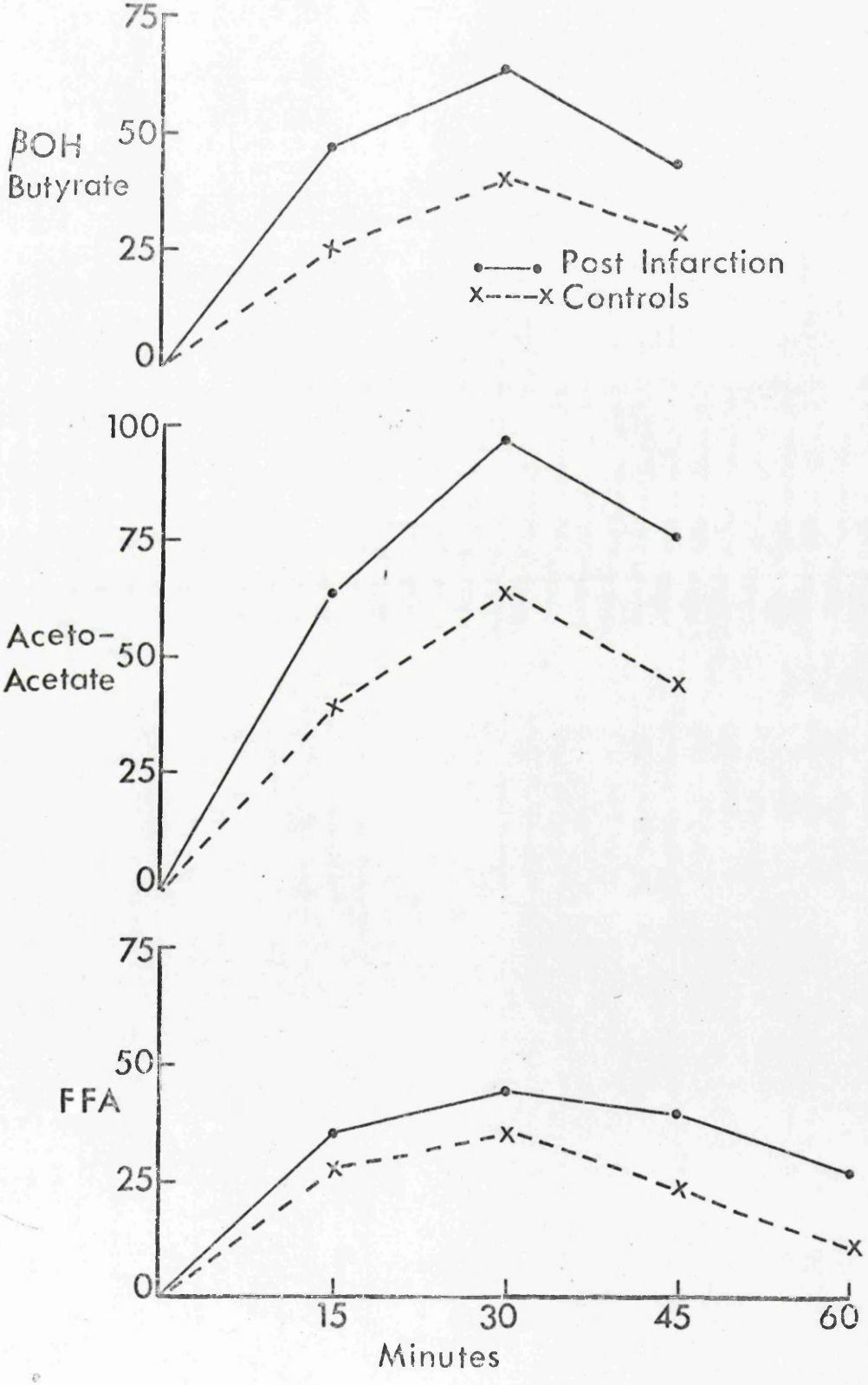


FIGURE 3

Percentage change in levels of FFA, Aceto-Acetate and βOH Butyrate after Smoking 2 normal cigarettes in control Subjects and those with Angina or PVD.

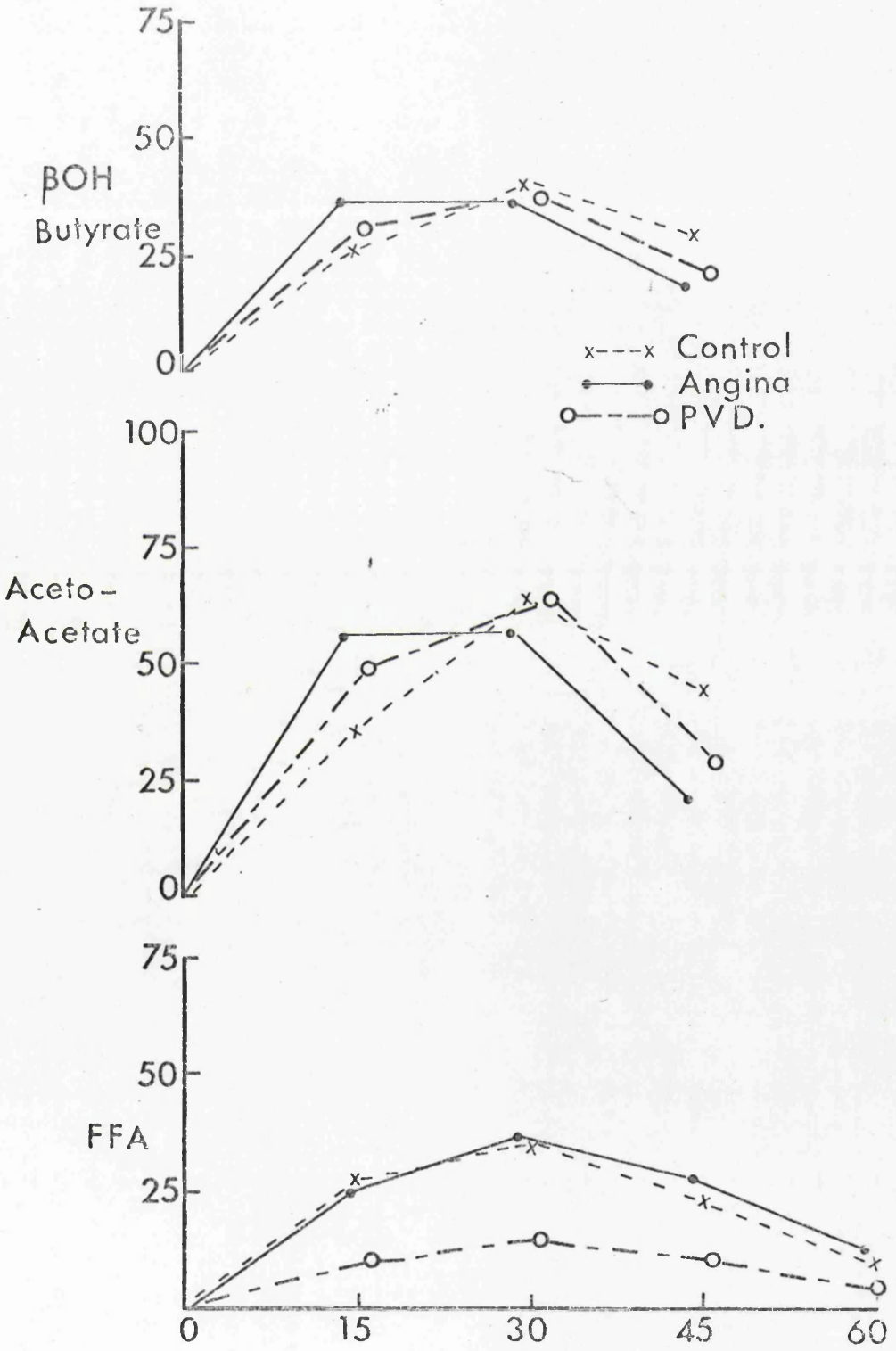


FIGURE 4

Increments in FFA and Ketone bodies after Smoking 2 Normal Cigarettes.

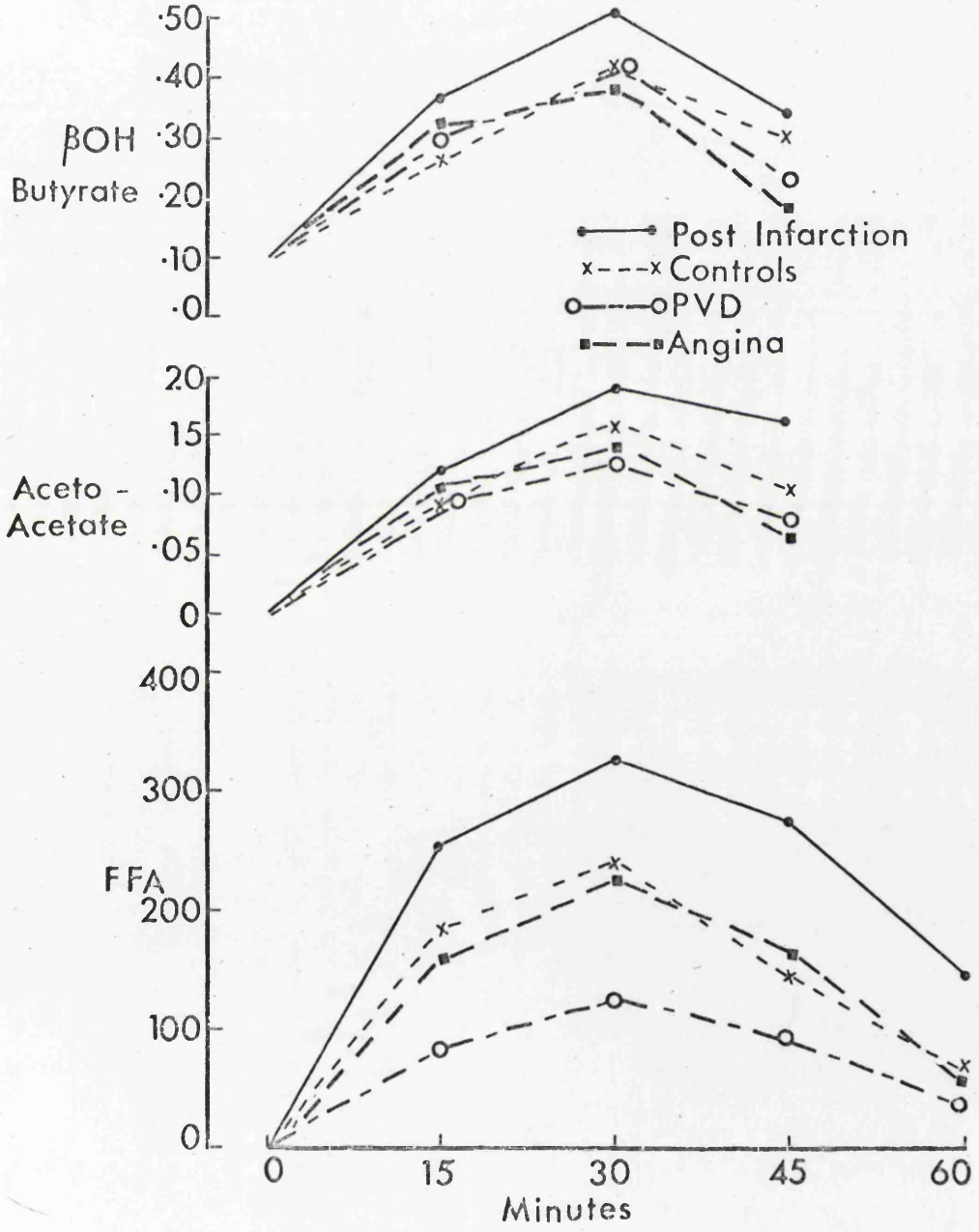
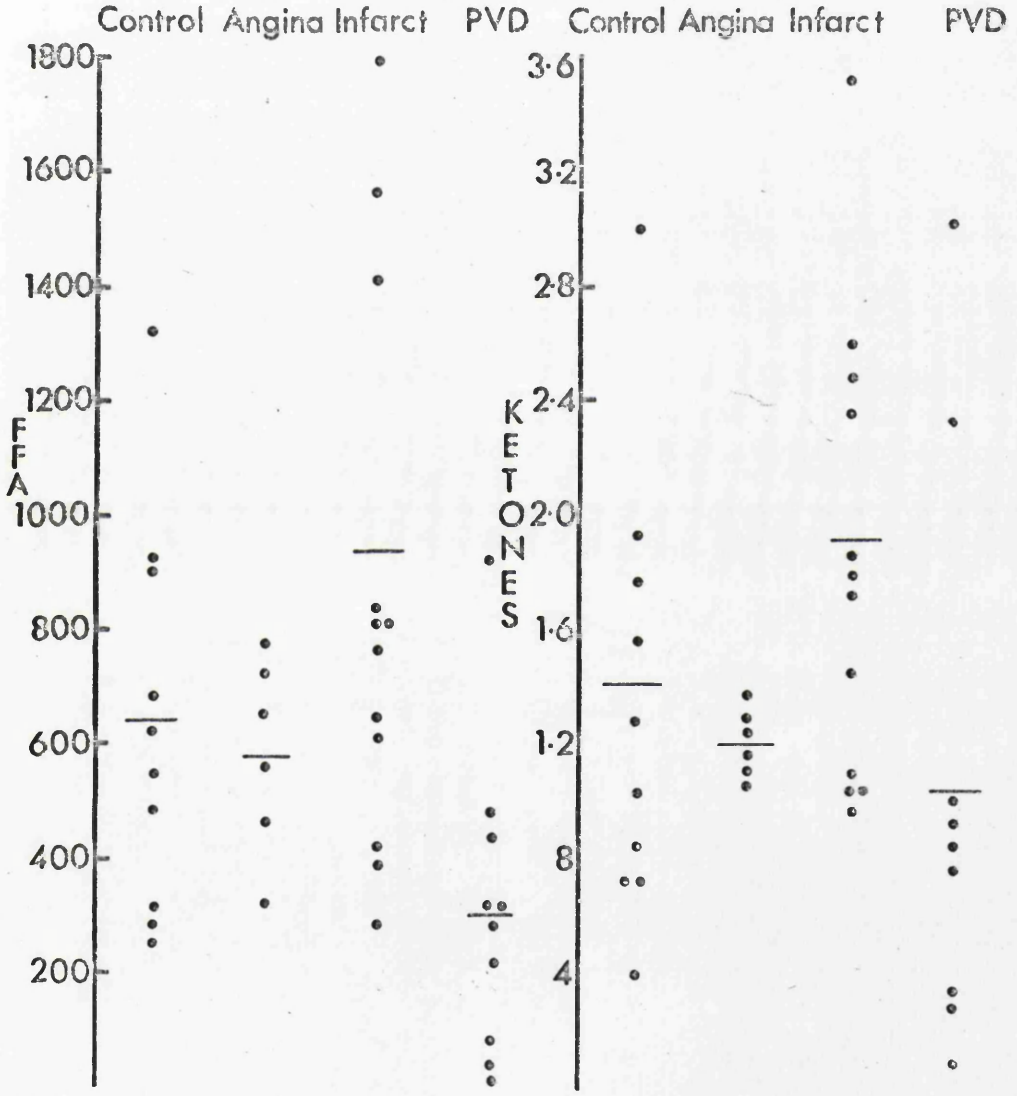


FIGURE 5

Relationship between total FFA Production and total Ketone Body Production after Smoking 2 Normal Cigarettes.



CHAPTER SIX

EFFECTS OF ADRENALINE ON CONTROL SUBJECTS AND THOSE WITH RECENT MYOCARDIAL INFARCTION

The effect of cigarette smoking is produced partly, at least, by sympathetic-adrenomedullary stimulation. Kingsbury and Jarrett (1967)[65] showed that the injection of a small amount of adrenaline subcutaneously could produce similar effects on FFA levels to that resulting from smoking 2 cigarettes. They also found that adrenaline produced a rise in blood sugar whereas cigarette smoking and that no change in insulin levels followed adrenaline administration. In view of the finding that post infarction subjects had a greater than control response to cigarette smoking it was decided to compare the effect of adrenaline administration in control subjects and in subjects who had sustained a recent myocardial infarction.

Study To assess the effect of the subcutaneous injection of 0.01 ml of 1: 10,000 adrenaline for kg body weight on levels of glucose, FFA, ketone bodies, insulin, cholesterol and triglyceride in control subjects and subjects 3 weeks after myocardial infarction.

There were 6 subjects in the control group. All were male, age range 44-59 years, mean 55 years. Their diagnoses were hypertension (3), mitral stenosis (1) and innocent systolic murmur(1). The 6 subjects studied 3 weeks after infarction were all male, age range 47-63 years, mean 55 years.

The conditions of investigation were as before with serial venous samples being obtained 15, 30, 45 and 60 minutes after the subcutaneous injection of the appropriate dose of adrenaline.

The results are shown in Tables 9A and B and in Figure 6. Previous studies had shown that the subcutaneous injection of saline produced small but not significant changes in any of the variables measured so that changes found after adrenaline injection are due to the adrenaline rather than merely to the noxious stimulus of an injection. In terms of the present study the main changes found were in FFA and ketone bodies. Significant increases ($P < 0.01$) in FFA and ketone bodies occurred in both the control and post infarction groups with the rise in FFA values in the post infarction group being significantly higher than the control response at T45 and T60. There was no significant difference between the groups in terms of ketone body formation.

Overall the results suggest that, for similar doses of adrenaline, the response in FFA is greater in those subjects who have recently sustained a myocardial infarction and that this enhanced response would be in keeping with the similar greater increases found in this group of subjects after smoking 2 normal cigarettes.

TIME	AGE	GLUCOSE (mg/100 ml) 0 15 30 45 60	FFA (ueq/1) 0 15 30 45 60	ACETO-ACETATE (mg/100 ml) 0 15 30 45	β OH BUTYRATE (mg/100 ml) 0 15 30 45
CONTROL	55 ± 6	95 93 95 97 95 ± 7 ± 8 ± 12 ± 7 ± 8	683 883 948 765 700 ± 68 ± 84 ± 116 ± 49 ± 60	0.21 0.28 0.29 0.25 ± .10 ± .14 ± .16 ± .12	.74 1.03 1.06 0.94 ± .31 ± .32 ± .34 ± .34
POST INFARCT	55 ± 7	89 89 87 92 88 ± 8 ± 4 ± 9 ± 11 ± 9	761 1232 1226 1056 916 ± 157 ± 217 ± 235 ± 249 ± 161	0.28 0.44 0.47 0.36 ± .13 ± .26 ± .21 ± .22	1.19 1.72 1.67 1.40 ± .27 ± .43 ± .47 ± .49

TIME	CHOLESTEROL (mg/100 ml) 0 15 30 45 60	TRIGLYCERIDE (mg/100 ml) 0 15 30 45 60	INSULIN 0 15 30 45 60
CONTROL	203 208 208 206 205 ± 36 ± 38 ± 35 ± 39 ± 39	106 106 107 104 104 ± 26 ± 26 ± 27 ± 25 ± 28	20 34 20 32 19 ± 13 ± 14 ± 11 ± 14 ± 11
POST INFARCT	214 215 219 218 215 ± 61 ± 58 ± 67 ± 62 ± 63	118 112 117 117 117 ± 26 ± 28 ± 28 ± 28 ± 26	30 31 28 32 31 ± 11 ± 11 ± 7 ± 11 ± 11

TABLE 9A EFFECT OF THE SUBCUTANEOUS INJECTION OF 0.01 µL OF 1:10,000 ADRENALINE PER KG BODYWEIGHT ON LEVELS OF GLUCOSE, FFA, KETONE BODIES, CHOLESTEROL AND INSULIN IN CONTROL SUBJECTS AND SUBJECTS 3 WEEKS AFTER MYOCARDIAL INFARCTION

Absolute value ± SD is shown

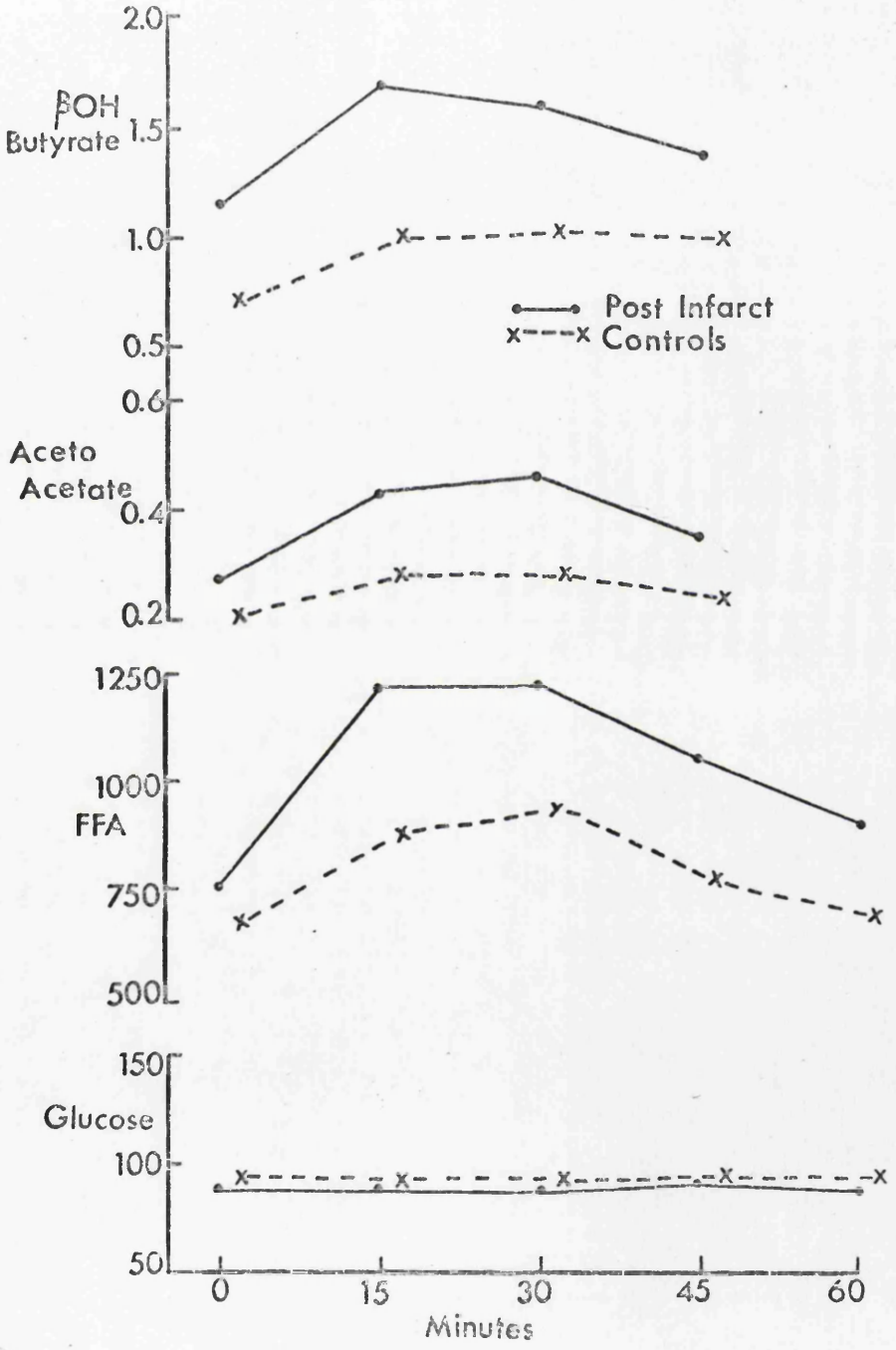
	FFA	ACETO-ACETATE	β OH BUTYRATE	INSULIN
TIME	15 30 45 60	15 30 45	15 30 45	15 30 45 60
CONTROL	48 40 14 2 ± ± ± ± 55 25 8 7	31 37 19 ± ± ± 20 13 15	33 38 22 ± ± ± 38 34 33	23 6 5 1 ± ± ± ± 23 17 29 17
POST INFARCT	67 67 46 25 ± ± ± ± 50 51 40 23	47 60 31 ± ± ± 28 45 37	40 38 17 ± ± ± 38 24 25	3 -2 7 12 ± ± ± ± 17 11 12 15

TABLE 9B EFFECT OF THE SUBCUTANEOUS INJECTION OF 0.01 uL OF 1:10,000 ADRENALIN FOR KG BODYWEIGHT ON LEVELS OF GLUCOSE, FFA, KETONE BODIES, CHOLESTEROL AND INSULIN IN CONTROL SUBJECTS AND SUBJECTS 3 WEEKS AFTER MYOCARDIAL INFARCTION

Percentage change ± SD is shown

FIGURE 6

Effect of 0·01 μl of 1:10,000 Adrenaline per kg body weight on levels of Glucose, FFA, Aceto-Acetate and βOH Butyrate.



CHAPTER SEVEN

RESPONSE TO CIGARETTE SMOKING IN THOSE WITH NORMAL LIPIDS AND THOSE WITH HYPERLIPOPROTEINAEMIA

There has been considerable interest over the past four years in the classification of lipid abnormalities - the hyperlipoproteinaemias - as proposed by Fredrickson, Levy and Lees (1967).[36] An extension to this classification was also suggested by Beaumont et al (1970).[12B] In general terms the classification separates subjects into those with normal lipids and those with Type 1-5 abnormalities. The commonest are Type II abnormality where total cholesterol is raised and triglyceride is normal and Type IV abnormality where total cholesterol is normal but triglyceride is raised. Normal values for cholesterol and triglyceride and the prevalence of the hyperlipoproteinaemias in male subjects at work in the West of Scotland have been reported (Lorimer, Cox, Greaves, Hawthorne, Jubb, Morgan and Lawrie 1972).[83] Epidemiological studies have indicated that hyperlipidaemia as shown by raised cholesterol or raised triglyceride, either singly or in combination, are among the major risk factors in the development of CHD (Blackburn 1970,[14] Carlson and Böttiger 1972).[18] It seemed appropriate to investigate the FFA and ketone body response to cigarette smoking in subjects with normal and abnormal lipids. The classification with hyperlipoproteinaemias was based on measurements of total cholesterol and triglyceride, facilities for ultracentrifugation of lipoproteins not being available.

Study A To assess the effect of smoking 2 non-tipped normal nicotine content cigarettes on levels of glucose, FFA, ketone bodies and insulin in subjects with normal lipid values and those with probable Type II or Type IV hyperlipoproteinaemia.

There were 13 male subjects in the normal lipid value group, age range 35-58 years, mean 48 years. There were 7 male subjects in the probable Type II group, age range 32-54, mean 42 years while in the probable Type IV group there were 6 male subjects, age range 43-59 years, mean 51 years.

The results are shown in Tables 10A and B and Figure 7. They indicate that a satisfactory differentiation between the groups is present in terms of cholesterol and triglyceride values. The resting basal values for FFA, ketone bodies and insulin all tended to be higher in the Type IV group - possibly reflecting the slightly increased bodyweight of this group. The responses to cigarette smoking were similar in each group with no significant differences between them being found. It had been felt that those with Type IV abnormality might have shown an enhanced release of FFA but this did not initially appear to be so. However, when increments of FFA were measured by subtracting the basal (T0) value from those found at T15, T30, T45 and T60 it was found that there was no difference between subjects with normal lipids and those with Type II abnormality but that those with Type IV had a significantly greater release ($P<0.01$) of FFA at 15, 30 and 45 minutes. The increases in ketone bodies were also higher in the Type IV group although satisfactory levels of significance were not obtained. These results are shwon

in Table 11. These results can be compared ~~to~~ with those reported by Nestel (1964) who found a significant relationship between plasma triglyceride concentration and the absolute and percentile increments in FFA that occurred after a 15 minute infusion of noradrenaline. When ketone body/FFA relationship are examined it can be seen that overall the ratio is similar in all groups. Thus the increased FFA production of type IV is associated with a corresponding ketone body increase.

TIME	AGE	GLUCOSE (mg/100 ml) 0 15 30 45 60	FFA (ueq/l) 0 15 30 45 60	ACETO-ACETATE (mg/100 ml) 0 15 30 45	β OH BUTYRATE (mg/100 ml) 0 15 30 45
NORMAL LIPIDS	48	91 ± 8, 93 ± 9, 94 ± 9, 95 ± 10, 92 ± 8	790 ± 217, 1007 ± 917, 1045 ± 236, 998 ± 264, 892 ± 217	0.25 ± .10, 0.36 ± .12, 0.40 ± .12, 0.35 ± .11	1.10 ± .30, 1.40 ± .30, 1.48 ± .33, 1.32 ± .32
TYPE II	42	94 ± 13, 100 ± 18, 99 ± 17, 97 ± 15, 98 ± 17	711 ± 211, 847 ± 198, 911 ± 291, 848 ± 314, 753 ± 248	0.24 ± .05, 0.34 ± .05, 0.38 ± .08, 0.38 ± .10	1.11 ± .21, 1.45 ± .37, 1.60 ± .48, 1.53 ± .54
TYPE IV	51	95 ± 16, 91 ± 15, 91 ± 12, 89 ± 14, 89 ± 14	903 ± 179, 1232 ± 325, 1323 ± 396, 1142 ± 223, 956 ± 197	0.36 ± .20, 0.53 ± .27, 0.59 ± .59, 0.54 ± .39	1.22 ± .56, 1.75 ± .45, 1.89 ± .78, 1.70 ± .79

TIME	INSULIN (units/ml) 0 15 30 45 60	CHOLESTEROL (mg/100 ml) 0	TRIGLYCERIDE (mg/100 ml) 0
NORMAL LIPIDS	27 ± 8, 27 ± 8, 26 ± 7, 29 ± 9, 28 ± 9	222 ± 28	119 ± 18
TYPE II	22 ± 7, 24 ± 10, 22 ± 11, 20 ± 6, 20 ± 6	302 ± 29	122 ± 29
TYPE IV	32 ± 11, 34 ± 11, 28 ± 6, 31 ± 11, 30 ± 5	259 ± 37	270 ± 23

TABLE 10A EFFECT OF SMOKING 2 NON-TIPPED CIGARETTES IN LEVELS OF GLUCOSE, FFA, KETONE BODIES AND INSULIN IN SUBJECTS WITH NORMAL LIPIDS AND IN THOSE WITH PROBABLE TYPE II OR TYPE IV HYPERLIPOPROTEINAEMIA

Absolute value ± SD is shown

TIME	FFA 15 30 45 60	ACETO-ACETATE 15 30 45	β OH BUTYRATE 15 30 45
NORMAL LIPIDS	33 38 28 20 ± ± ± ± 25 37 43 39	42 56 41 ± ± ± 24 25 34	31 38 23 ± ± ± 24 19 21
TYPE II	22 29 21 7 ± ± ± ± 15 17 19 8	43 65 70 ± ± ± 26 44 58	31 45 39 ± ± ± 30 40 40
TYPE IV	33 43 26 6 ± ± ± ± 20 24 17 4	53 55 45 ± ± ± 29 40 40	53 63 40 ± ± ± 42 71 49

TABLE 10B EFFECT OF SMOKING 2 NON-TIPPED CIGARETTES IN LEVELS OF GLUCOSE, FFA, KETONE BODIES AND INSULIN IN SUBJECTS WITH NORMAL LIPIDS AND IN THOSE WITH PROBABLE TYPE II OR TYPE IV HYPERLIPOPROTEINAEMIA

Percentage changes ± SD is shown

GROUP	F.F.A. INCREASE ueq/l				ACETO-ACETATE INCREASE mg/100 ml			β OH BUTYRATE INCREASE mg/100 ml		
TIME	15	30	45	60	15	30	45	15	30	45
NORMAL LIPIDS	218 +- 137	257 +- 182	218 +- 185	121 +- 186	0.09 +- .04	0.13 +- .05	0.08 +- .06	0.31 +- .20	0.38 +- .17	0.23 +- .17
TYPE II	136 +- 83	215 +- 116	112 +- 77	59 +- 64	0.10 +- .05	0.15 +- .08	0.16 +- .10	0.34 +- .34	0.50 +- .40	0.44 +- .47
TYPE IV	332 +- 159	405 +- 232	240 +- 134	55 +- 40	0.17 +- .12	0.23 +- .12	0.20 +- .19	0.53 +- .41	0.69 +- .60	0.49 +- .50

TABLE 11 INCREMENTS OF F.F.A. AND KETONE BODIES FOUND AFTER SMOKING 2 NON TIPPED CIGARETTES IN SUBJECTS WITH NORMAL LIPIDS, PROBABLE TYPE II AND PROBABLE TYPE IV HYPERLIPOPROTEINAEMIA

Mean ± SD is shown

FIGURE 7

Effect of Smoking 2 normal cigarettes on levels of Glucose FFA, Aceto-Acetate and βOH Butyrate in Subjects with Normal and Abnormal Lipids.

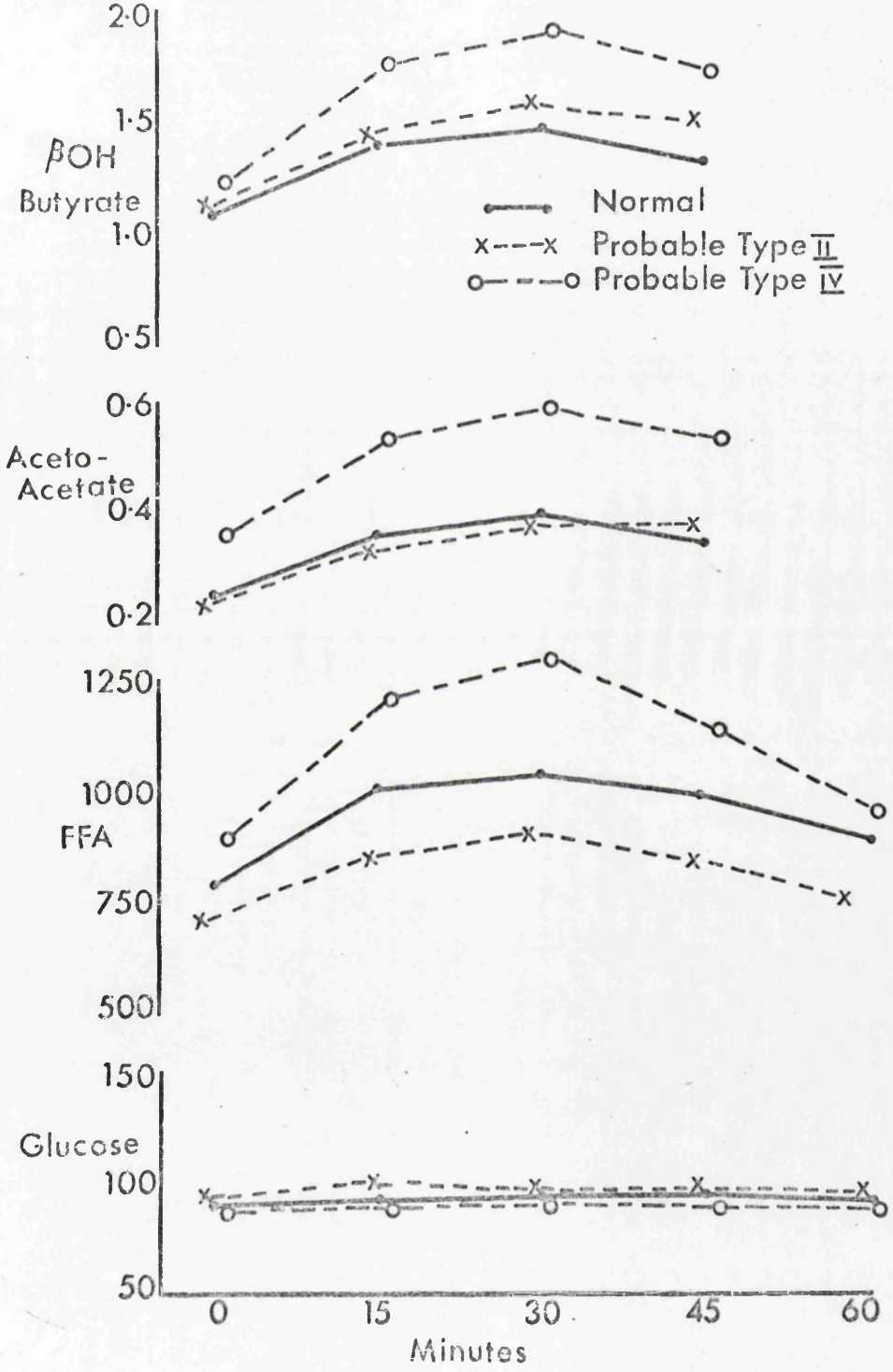
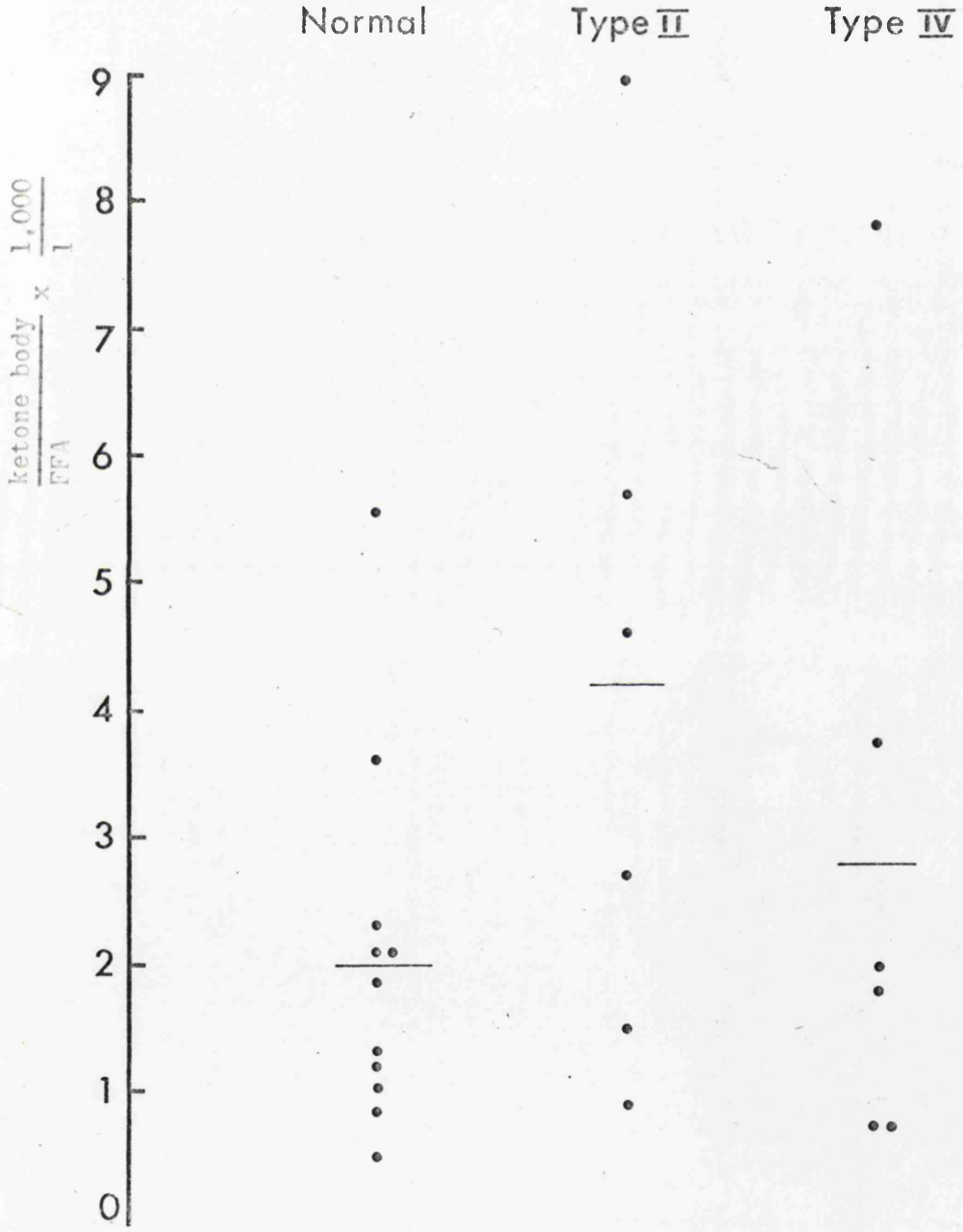


FIGURE 8

Ketone Body/FFA Relationship in Subjects with Normal Lipids and Type II and Type IV Abnormality.



CHAPTER EIGHT

RESPONSE TO CIGARETTE SMOKING 1 YEAR AFTER MYOCARDIAL INFARCTION AND EFFECT OF BETA-ADRENORECEPTOR BLOCKADE ON THIS RESPONSE

Previous reports have shown that shortly after a myocardial infarction there is an enhanced release of FFA in response to smoking. Published work has not indicated whether or not this is a permanant finding nor whether or not it can be pharmacologically prevented. Studies were therefore undertaken in subjects one year after documented myocardial infarction, and in addition the effect of beta-adrenoreceptor blockade was investigated.

Study A To assess the effect of smoking 2 non-tipped normal nicotine content cigarettes on levels of FFA and ketone bodies in subjects one year after myocardial infarction and to compare the effects of IV propranolol and placebo on levels of FFA and ketone bodies after smoking 2 normal cigarettes.

Seven male subjects (age range 36-56 years, mean 44 years) were studied approximately one year after myocardial infarction. All had made an apparently good recovery and had resumed smoking 15-20 cigarettes daily. The conditions of investigation rose as previously described with subjects being admitted the day before investigation and fasted overnight.

Six male subjects were studied after the IV administration of propranolol or placebo. All had sustained a myocardial infarction one year before and had resumed smoking. Each was randomly allocated to

either propranolol or placebo on day 1 with the other being given on day 2. A baseline sample of venous blood was obtained then either propranolol in a dose of 0.05 mg/kg bodyweight or 10 ml saline were injected slowly IV. Subjects then smoked 2 normal cigarettes with venous samples being obtained at the times listed.

The results are shown in Tables 12A and Figure 9. They indicate that one year after myocardial infarction the response to cigarette smoking resembles that found in control subjects. It should also be noted that the basal levels for FFA and ketone bodies are similar in the post infarction and control group. The enhanced fasting levels of FFA found in subjects 3 weeks after infarction was no longer present one year after infarction. Nor was there any tendency for increased FFA release after smoking. These subjects had made a good recovery and were neither in cardiac failure nor experiencing angina. These results suggest that the increase release of FFA found after infarction is a transient phenomenon.

Propranolol is a non-selective beta-adrenoreceptor blocking agent ie it acts on adrenergic receptors other than those found in the heart. It is widely used in the treatment of cardiac dysrhythmias, angina due to CHD and systemic hypertension. It has also been suggested that beta-adrenoreceptor blockade may have prophylactic value in the prevention of recurrence of myocardial infarction. The effect on the release of FFA and ketone bodies after cigarette smoking is shown in Table 13 and Figure 10. Intravenous propranolol completely abolished the FFA response to cigarette smoking at all time intervals when compared to placebo. There was also a marked reduction in ketone body levels although it should be noted that a small response persisted. Overall, however, the changes in

ketone body levels did not reach significant levels.

It seems likely, therefore, that propranolo is effective in reducing catecholamine induced FFA ~~flow~~ increase and this action could have considerable clinico-pharmacological significance.

	FFA (ueq/l)					ACETO-ACETATE (mg/100 ml)				BOH BUTYRATE (mg/100 ml)			
TIME	0	15	30	45	60	0	15	30	45	0	15	30	45
1 YEAR POST INFARCT	696 ± 199	821 ± 176	882 ± 229	864 ± 272	791 ± 228	0.29 ± .18	0.44 ± .19	0.52 ± .22	0.50 ± .32	1.15 ± .56	1.63 ± .73	2.06 ± 1.01	1.88 ± 1.08

TABLE A Absolute values

FFA				ACETO-ACETATE			BOH BUTYRATE		
15	30	45	60	15	30	45	15	30	45
21 ± 15	28 ± 14	25 ± 13	15 ± 10	57 ± 21	70 ± 26	61 ± 26	45 ± 31	67 ± 33	55 ± 28

TABLE B Percentage change

TABLES 1 2A and B EFFECT OF SMOKING 2 NON-TIPPED CIGARETTES ON LEVELS OF FFA AND KETONE BODIES IN SUBJECTS ONE YEAR AFTER MYOCARDIAL INFARCTION

TIME	FFA (ueq/l) 0	15	30	45	60	ACETO-ACETATE (mg/100 ml) 0	15	30	45	β OH BUTYRATE 0	15	30	45
PLACEBO	739 ± 178	852 ± 172	928 ± 213	919 ± 252	850 ± 190	0.31 ± .19	0.46 ± .20	0.55 ± .23	0.54 ± .24	1.21 ± .59	1.80 ± .77	2.28 ± .91	2.07 ± .92
PROPRANOLOL	725 ± 174	681 ± 162	636 ± 144	698 ± 180	688 ± 185	0.28 ± .16	0.34 ± .22	0.35 ± .27	0.34 ± .25	1.16 ± .50	1.33 ± .76	1.43 ± .75	1.41 ± .81

TABLE 13 EFFECT OF 0.05 MG/KG OF PROPRANOLOL IV ON LEVELS OF FFA AND KETONE BODIES AFTER SMOKING 2 NORMAL CIGARETTES IN SUBJECTS ONE YEAR AFTER MYOCARDIAL INFARCTION

FIGURE 9

Effect of Smoking 2 Normal cigarettes on levels of FFA, Aceto-Acetate and βOH Butyrate in Control subjects and those 3weeks and 1year after Myocardial Infarction.

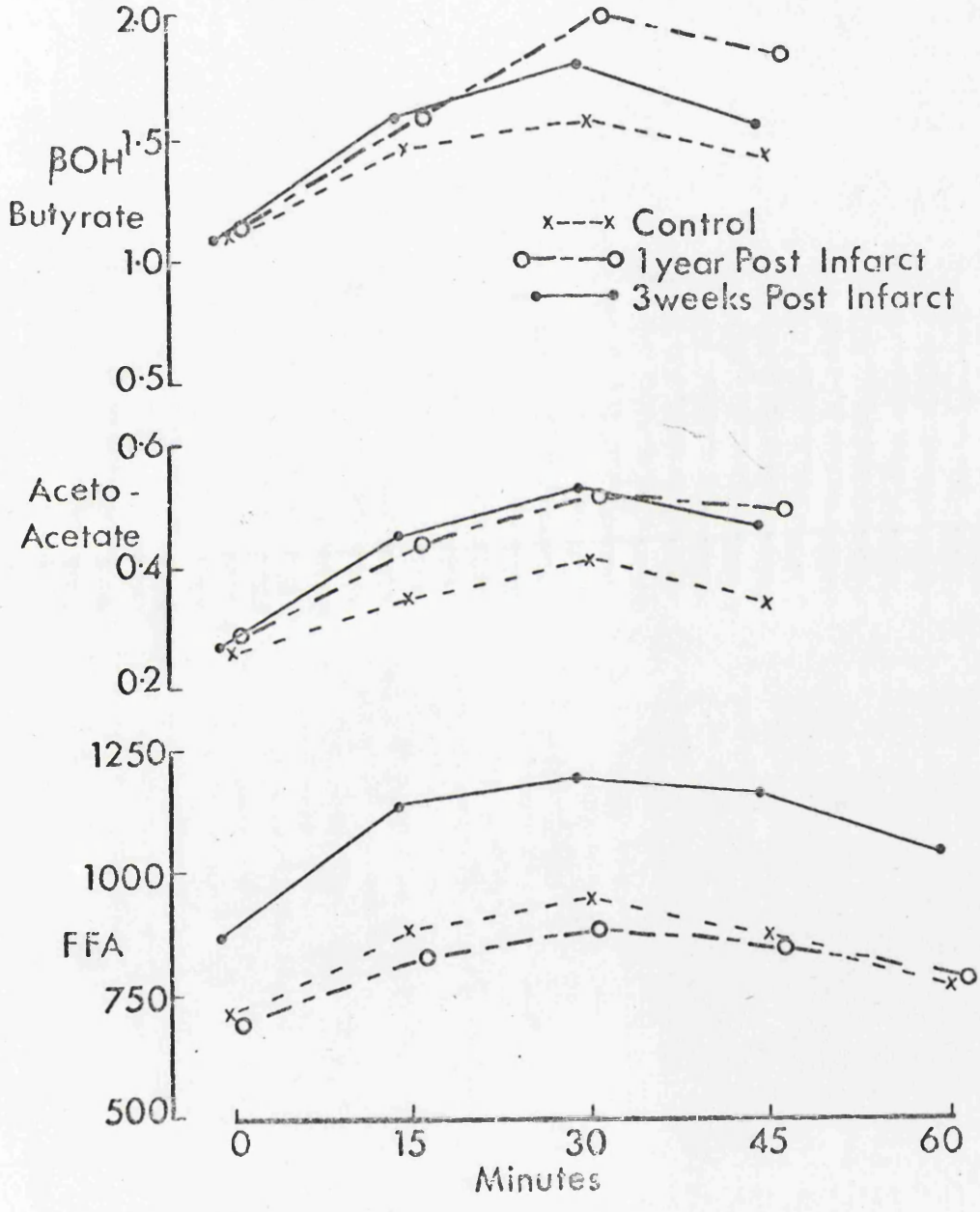
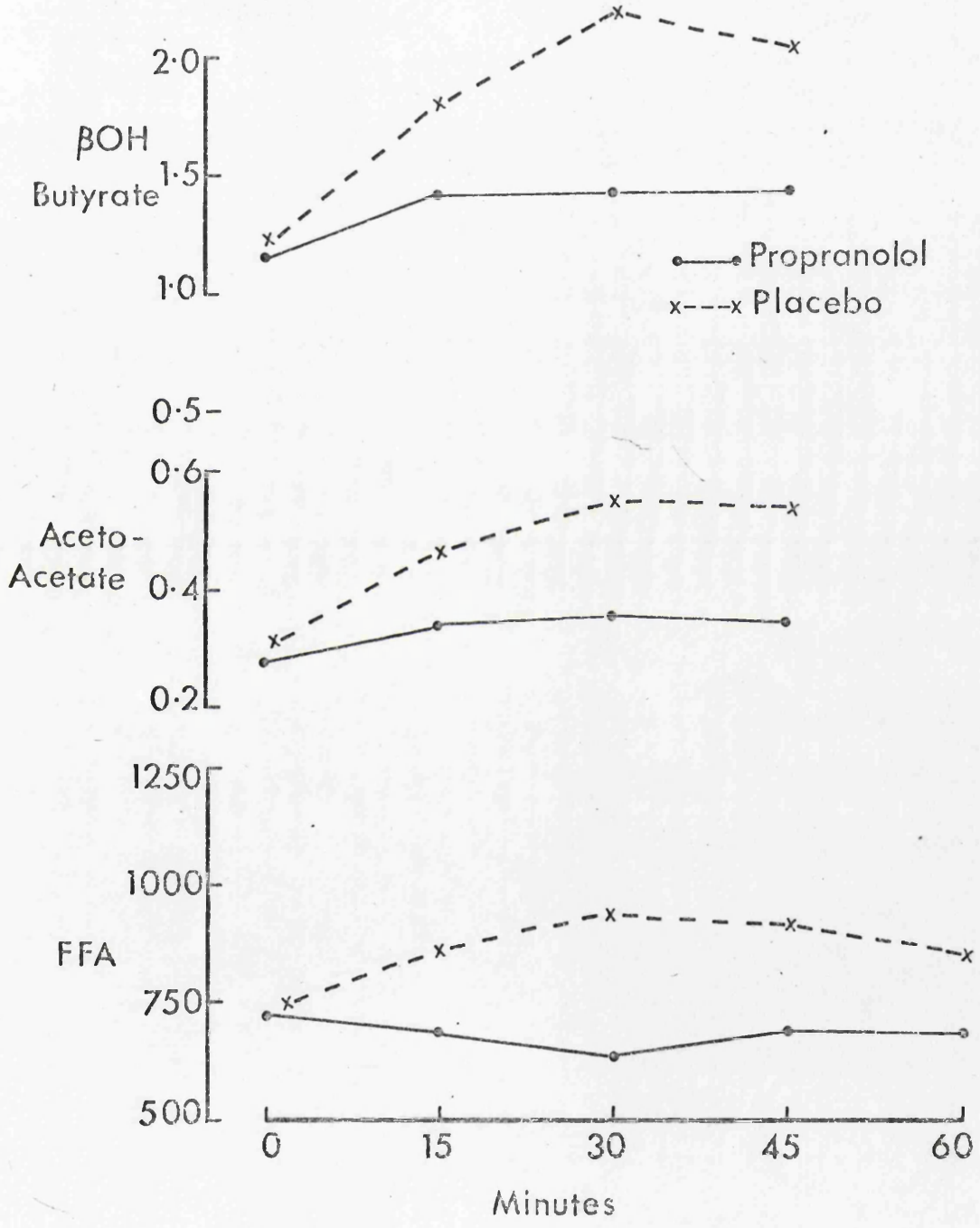


FIGURE 10

Effect of Propranolol and Placebo on level of FFA and Ketone Bodies after Smoking 2 Normal Cigarettes.



CHAPTER NINE

EFFECT OF SMOKING 6 CIGARETTES OVER 3 HOURS IN THOSE WITH AND WITHOUT OVERT VASCULAR DISEASE

The studies described in earlier chapters indicate that the smoking of 2 normal cigarettes is sufficient to produce changes in levels of FFA and ketone bodies but insufficient to cause any alteration in levels of glucose or insulin. It was felt that smoking 2 cigarettes might be an inadequate stimulus to produce changes in these variables and so the investigation was repeated after smoking 6 normal nicotine content cigarettes over a 3 hour period. It was not felt justifiable to ask those who had sustained a recent infarction to smoke this number of cigarettes and so the study was restricted to control subjects and subjects with either angina or PVD.

Study — To assess the effect of smoking 6 low nicotine content cigarettes over a 3 hour period and to compare the effect of smoking 6 normal nicotine content cigarettes smoked 3 hours on levels of FFA, ketone bodies, glucose insulin, cholesterol and triglyceride in control subjects and those with angina or PVD.

There were 5 subjects in the group smoking low nicotine content cigarettes. All were male (age range 37-58 years, mean 48 years). Five male subjects (age range 37-64 years, mean 49 years) smoking 6 normal cigarettes. None had a history of myocardial infarction, angina or PVD. There were 6 male subjects in the PVD group (age range

45-67 years, mean 53 years). All had angiographically demonstrated vascular disease of the lower limbs but gave no history of angina or myocardial infarction. The 6 male subjects with angina (age range 39-53 years, mean 45 years) all had abnormal resting electrocardiograms and coronary angiograms demonstrating significant disease.

Subjects were fasted overnight. An intravenous cannula was inserted as previously described as a baseline sample obtained. Subjects then smoked 1 low nicotine or 1 normal nicotine content cigarette every 30 minutes for 3 hours and a further venous sample was obtained at the end of this time

The results are shown in Tables 14A and B and Figure 11. When the subjects are examined in groups, the significant changes occurred in FFA and ketone bodies. Smoking low nicotine content cigarettes failed to produce any change in the variables measured, but after smoking 6 normal cigarettes FFA values increased by 36-42 per cent. While aceto-acetate and B hydroxybutyrate increased by 102-150 and 101-128 per cent respectively. There was no difference in the level of response between control subjects and those with overt CHD or PVD. This could suggest that basically all cigarette smoking subjects respond to heavy cigarette smoking in much the same way. Such effects may of course be more harmful in those with an already compromised vascular tree. There was no significant change overall in mean levels of insulin, cholesterol or triglyceride.

It was also decided to compare each individual's response to smoking. There were 2 main groups, those whose blood sugar rose by more than 15 mg per 100 ml and those in whom the blood glucose rose

by less than this amount or even fell. Individual results are shown in Table 15.

In the group showing a rise in blood glucose, the mean level rose from 91 to 117 mg/100 ml while in the other the value fell from 94 to 82 mg/100 ml. This difference in glucose response was not reflected by differences in FFA or ketone body values. These variables rose by a comparable amount in both groups. Thus the FFA level rose from 860 to 1190 meq/l in those with a rise in glucose, whereas those with a fall in glucose rose from 859 to 1199 meq/l with similar changes occurring in the group showing a fall in blood sugar. Somewhat surprisingly, the insulin response was relatively unchanged. Mean levels in both groups rose slightly but did not alter significantly.

GROUP	AGE	TYPE OF CIGARETTE	GLUCOSE mg/100 ml		FFA ueq/l		ACETO-ACETATE mg/100 ml		β OH BUTYRATE mg/100 ml		INSULIN units/ml		CHOLESTEROL mg/100 ml		TRIGLYCERIDE mg/100 ml	
			0	180	0	180	0	180	0	180	0	180	0	180	0	180
NORMAL + PVD	48 ± 10	LOW NICOTINE	93 ± 12	94 ± 10	878 ± 88	875 ± 75	0.23 ± .04	0.24 ± .05	1.20 ± .19	1.12 ± .21	29 ± 12	28 ± 6	247 ± 31	244 ± 32	134 ± 57	138 ± 59
CONTROL	50 ± 10	NORMAL	98 ± 16	109 ± 20	817 ± 96	1110 ± 127	0.23 ± .04	0.53 ± .07	1.10 ± .15	2.50 ± .41	26 ± 7	27 ± 9	262 ± 28	261 ± 27	124 ± 57	123 ± 59
PVD	52 ± 13	NORMAL	87 ± 10	102 ± 25	811 ± 55	1126 ± 116	0.21 ± .06	0.51 ± .09	1.02 ± .25	2.11 ± .21	25 ± 4	36 ± 25	232 ± 32	234 ± 37	119 ± 26	122 ± 22
ANGINA	45 ± 5	NORMAL	94 ± 9	99 ± 22	923 ± 130	1179 ± 139	0.30 ± .11	0.57 ± .13	1.31 ± .44	2.50 ± .53	20 ± 10	23 ± 15	266 ± 40	270 ± 43	130 ± 42	126 ± 44

TABLE 14A EFFECT OF SMOKING 6 LOW NICOTINE OR 6 NORMAL NICOTINE CONTENT CIGARETTES OVER 3 HOURS ON LEVELS OF GLUCOSE, FFA, KETONE BODIES, INSULIN, CHOLESTEROL AND TRIGLYCERIDE IN CONTROL SUBJECTS AND IN SUBJECTS WITH PVD OR ANGINA

Absolute value ± SD is shown

GROUP	TYPE OF CIGARETTE	GLUCOSE	FFA	ACETO-ACETATE	β OH BUTYRATE	INSULIN
NORMAL + PVD	LOW NICOTINE	2 ± 5	-0.2 /	6 ± 19	1 ± 9	7 ± 47
CONTROL	NORMAL	13 ± 28	36 ± 8	128 ± 20	128 ± 40	6 ± 23
PVD	NORMAL	15 ± 31	39 ± 17	150 ± 48	116 ± 56	39 ± 101
ANGINA	NORMAL	5 ± 16	42 ± 12	102 ± 56	101 ± 56	25 ± 43

TABLE 14B EFFECT OF SMOKING 6 LOW NICOTINE OR 6 NORMAL NICOTINE CONTENT CIGARETTES OVER 3 HOURS ON LEVELS OF GLUCOSE, FFA, KETONE BODIES, INSULIN, CHOLESTEROL AND TRIGLYCERIDE IN CONTROL SUBJECTS AND IN SUBJECTS WITH PVD OR ANGINA

Percentage change ± SD is shown

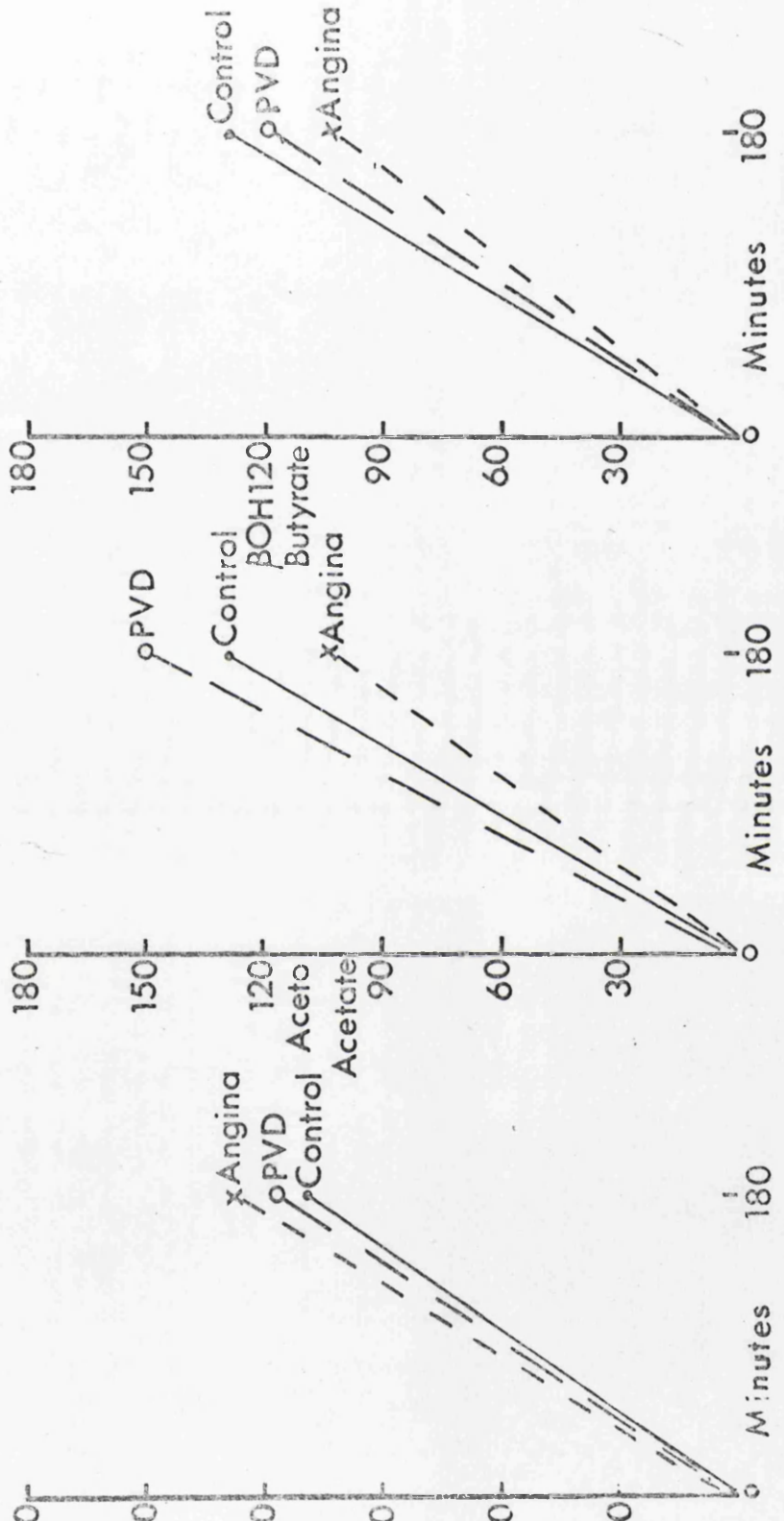
TABLE 15 INDIVIDUAL RESPONSES TO SMOKING 6 NORMAL CIGARETTES. COMPARISON OF THOSE WHOSE BLOOD SUGAR ROSE WITH THOSE IN WHOM BLOOD SUGAR FELL OR REMAINED UNCHANGED

GROUP	AGE	GLUCOSE		FFA		ACETO-ACETATE		βOH BUYRATE		INSULIN	
		0	180	0	180	0	180	0	180	0	180
CONTROL	45	100	135	748	1070	0.27	0.62	1.17	2.20	25	21
"	37	73	106	926	1294	0.20	0.43	1.10	2.30	34	37
"	64	100	117	895	1092	0.23	0.47	1.04	2.10	18	16
PVD	45	89	107	832	1001	0.12	0.35	0.65	1.89	26	40
"	48	74	120	740	1184	0.20	0.60	1.17	2.02	24	81
"	44	79	100	788	1209	0.27	0.54	1.30	2.00	25	12
"	67	103	131	883	1283	0.24	0.50	1.20	1.92	32	33
ANGINA	44	106	125	952	1274	0.39	0.62	1.30	1.92	10	13
"	41	98	113	980	1308	0.32	0.58	1.70	2.50	25	30
		91	117	860	1190	0.25	0.52	1.18	2.09	24	31
CONTROL	48	119	106	699	943	0.23	0.54	1.06	2.70	31	33
"	54	100	80	817	1150	0.23	0.59	1.10	3.10	21	30
PVD	55	88	75	768	1070	0.27	0.55	1.08	2.30	27	33
"	58	91	69	856	1009	0.20	0.51	0.94	2.38	19	14
ANGINA	39	91	76	1094	1805	0.47	0.78	1.97	3.30	14	12
"	46	82	70	972	1324	0.16	0.43	0.80	2.07	12	27
"	53	87	96	810	1090	0.23	0.43	1.04	2.20	35	37
		94	82	859	1199	0.25	0.55	1.13	2.58	23	27

FIGURE 11

Effect of Smoking 6 Normal Cigarettes in control subjects and those with Angina or PVD.

Percentage Change in FFA. and Ketone Bodies.



CHAPTER TEN

EFFECT OF CIGARETTE SMOKING ON URINARY CATECHOLAMINES

Cigarette and cigar smoking in man, as well as the injection of nicotine in experimental animals, have been shown to increase urinary catecholamine excretion. Kershbaum (1968)[58] found a rise in total catecholamines with noradrenaline increasing less than adrenaline, while Watts (1960)[132] reported a predominant increase in urinary adrenaline rather than noradrenaline following heavy cigarette smoking in healthy young adult males.

A series of studies were therefore undertaken to measure changes in urinary adrenaline and noradrenaline in subjects with and without vascular disease produced as a consequence of cigarette smoking. The effect of subcutaneous adrenaline on levels of FFA and ketone bodies was also studied.

Study A To assess the effects of cigarette smoking on levels of urinary catecholamines.

1. The effect of smoking 2 low nicotine or 2 normal nicotine content cigarettes in control subjects and in subjects 3 weeks after myocardial infarction.
2. The effect of smoking 6 low nicotine or 6 normal nicotine content cigarettes in control subjects and in subjects with angina or PVD.

Control subjects were all male and had no history or clinical features of CHD or PVD. Those subjects who had sustained a recent myocardial infarction were all male, had smoked more than 20 cigarettes

daily before infarction and had resumed smoking 5-10 cigarettes daily.

The study was conducted as follows. Subjects were fasted overnight. In the morning the bladder was emptied and subjects then either did not smoke or smoked 2 low nicotine or 2 normal nicotine content cigarettes for 10 minutes each with a 5 minute interval between. Two hundred ml of water were drunk each hour and after 2 hours the bladder was again emptied and the urine collected into a small volume of concentrated hydrochloric acid. Measurement of urinary adrenaline and noradrenaline were done as described by Lorimer, McFarlane, Provan, Duffy and Lawrie (1971).[84]

A similar protocol was followed for smoking 6 cigarettes. These were smoked over a 3 hour period with the bladder being emptied beforehand and 200 ml of water again being drunk hourly.

The results (Tables 16A and B, Figure 12) show that there is no apparent difference in resting urinary catecholamine levels in control subjects and subjects following a recent myocardial infarction. Such subjects have significantly different resting FFA levels and the failure of urinary catecholamines to differentiate between them could be a reflection of the small amount of the body's catecholamine pool that is broken down and excreted in the urine so that small changes may not be detectable by the technique used. There was no evidence that control subjects increased urinary catecholamines after smoking 2 low nicotine or 2 normal cigarettes. Nor was there any tendency (given the limitations of urinary catecholamine measurements) for post infarction subjects to have an increased urinary catecholamine

excretion after smoking 2 normal cigarettes although this stimulus had previously been shown to cause an enhanced release of FFA. The effect of smoking 6 normal cigarettes was not investigated on post infarction subjects. It was felt undesirable for them to smoke in such a concentrated fashion so soon after their acute episode. Smoking 6 normal cigarettes resulted in a significant (P 0.01) increase in both urinary adrenaline and noradrenaline in control subjects, those with PVD and those with CHD manifest by angina. In all groups the increase was of a similar amount. No group had a greater response than another and in addition changes in noradrenaline and adrenaline were similar among the groups.

Overall therefore it would appear that changes in sympathetic-adrenomedullary responses produced by cigarette smoking can be reflected in urinary catecholamine levels provided the stimulus is sufficient. The response is similar in all the clinical situations studied. The response to smoking 6 cigarettes reflects the similarity of FFA and ketone body level changes after similar exposure in subjects with PVD or angina.

GROUP	NO	AGE	TYPE OF CIGARETTE	BEFORE SMOKING			AFTER SMOKING		
				ADRENALINE	NORADRENALINE	TOTAL	ADRENALINE	NORADRENALINE	TOTAL
CONTROL + POST INFARCT	7	43 ± 12	LOW NICOTINE	3.3 ± 1.3	11.7 ± 3.1	15.0	2.5 ± 1.8	11.7 ± 3.5	14.2
CONTROL	7	39 ± 12	NORMAL	2.5 ± 1.5	10.3 ± 2.4	12.8	2.2 ± 1.4	10.5 ± 2.4	12.7
POST MYO INFARCT	7	51 ± 4	NORMAL	2.8 ± 1.4	14.0 ± 2.6	16.8	3.3 ± 1.6	13.8 ± 2.6	17.1

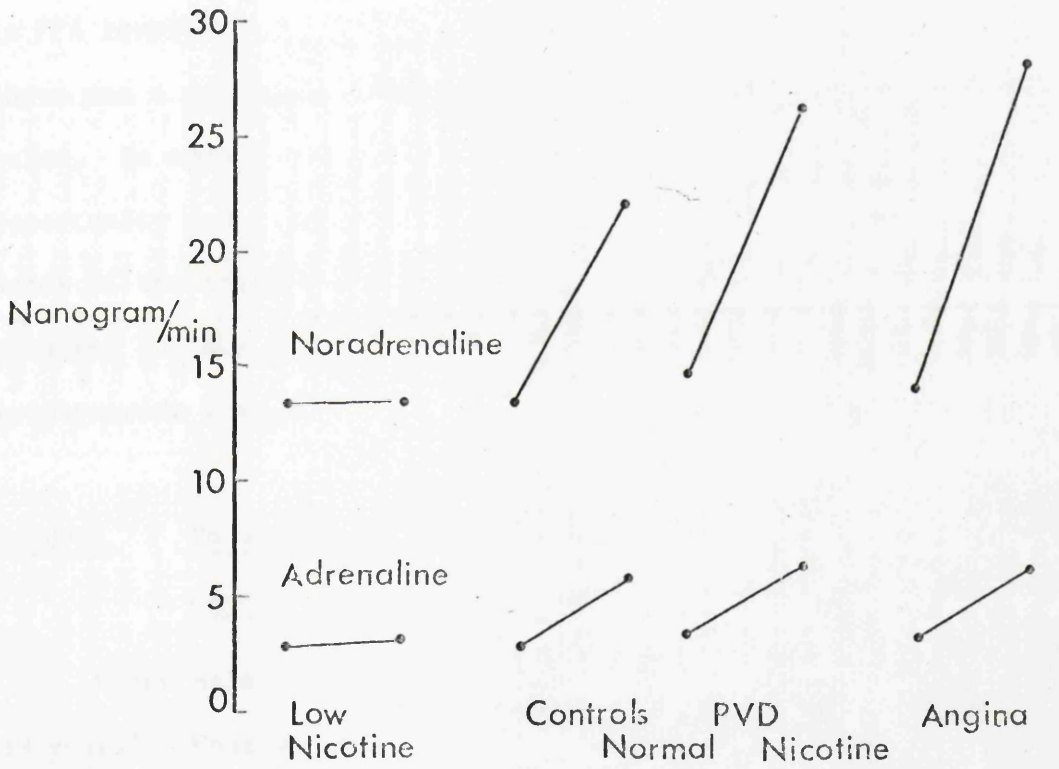
TABLE 16A EFFECT OF SMOKING 6 NORMAL CIGARETTES ON URINARY CATECHOLAMINES

GROUP	NO	AGE	TYPE OF CIGARETTE	BEFORE SMOKING			AFTER SMOKING		
				ADRENALINE	NORADRENALINE	TOTAL	ADRENALINE	NORADRENALINE	TOTAL
CONTROL	6	41 ± 14	LOW NICOTINE	2.7 ± 1.6	13.4 ± 3.2	16.1	2.9 ± 1.8	13.8 ± 4.0	16.7
CONTROL	6	41 ± 14	NORMAL	2.7 ± 1.6	13.4 ± 3.2	16.1	5.7 ± 2.0	22.3 ± 6.3	28.0
PVD	6	50 ± 10	LOW NICOTINE	3.4 ± 1.4	14.9 ± 4.1	18.3	2.8 ± 1.5	14.8 ± 4.3	17.6
PVD	6	50 ± 10	NORMAL	3.4 ± 1.4	14.9 ± 4.1	18.3	6.3 ± 2.1	26.6 ± 7.1	32.9
ANGINA	6	46 ± 8	NORMAL	3.1 ± 1.4	14.0 ± 3.9	17.1	6.3 ± 2.4	28.1 ± 6.8	34.4

TABLE 16B EFFECT OF SMOKING 6 NORMAL CIGARETTES IN URINARY CATECHOLAMINES

FIGURE 12

Urinary Catecholamine levels after Smoking 6 Low or 6 Normal Nicotine Content Cigarettes.



CHAPTER ELEVEN

EFFECT OF CIGARETTE SMOKING ON INDIVIDUAL FREE FATTY ACID AND TRIGLYCERIDE FATTY ACID LEVELS

The studies outlined in previous chapters have shown that FFA values increase after cigarette smoking. This change is found in all subjects although the extent of the response may vary. It was decided to further investigate this change by measuring levels of individual fatty acids to determine whether or not the overall change in FFA levels was due to a general increase in FFA values or whether there was a selective increase in one or more of the individual fatty acids. In addition to measuring the levels of individual FFA the opportunity was also taken to measure levels of individual fatty acids of the triglyceride fraction to assess whether or not changes occurred in them as a consequence of smoking even although total triglyceride levels did not appear to change.

Study A — To assess the effect of smoking 6 normal cigarettes on FFA and triglyceride fatty acid patterns.

Eight male subjects were studied (age range 36-62 years, mean 49 years). Four subjects had CHD as manifest by angina, 2 had PVD and 2 had indiopathic hypertension. Subjects were fasted overnight. Following insertion of a venous cannula, a 30 ml sample was taken with lithium heparin. Each subject then smoked 1 normal cigarette every 30 minutes for 3 hours, following which a further venous sample was obtained. Plasma FFA and triglyceride fatty acid patterns were

measured by their layer and gas liquid chromatography as described under methods.

C14 = myristate C16 = palmitate C16.1 = palmitoleate

C18 = stearate C18.1 = oleate C18.2 = linoleate

The results are shown in Tables 17A and B.

The principal fatty acids found in each fraction were palmitate and the unsaturated oleate. No change was found in the proportions of individual fatty acids after smoking either in terms of FFA or triglyceride fatty acids.

It seems likely that the increase in FFA found after cigarette smoking affects all fatty acids and not one or two selectively. It also seems likely that the absence of an effect on triglyceride values from cigarette smoking does not conceal any change in the fatty acid moieties of the triglyceride fraction.

SUBJECT	BEFORE SMOKING						AFTER SMOKING					
	C14	16	16.1	18	18.1	18.2	C14	16	16.1	18	18.1	18.2
1	1.6	35.5	-	18.5	37.0	6.8	6.1	27.0	-	6.5	60.2	-
2	5.8	43.0	8.2	8.2	30.1	4.3	7.2	38.4	4.6	10.0	34.6	5.0
3	5.4	47.0	6.2	8.9	29.4	2.7	7.1	54.6	7.7	7.9	27.6	4.1
4	7.6	41.5	13.4	5.1	27.8	4.2	7.7	40.6	11.6	3.6	31.6	4.6
5	9.5	44.1	7.7	8.3	26.2	4.0	5.3	46.7	10.4	6.5	27.0	3.8
6	3.5	44.7	8.7	6.6	30.5	5.8	4.6	50.0	7.3	10.2	23.0	4.5
7	8.8	52.8	4.8	10.4	17.2	6.0	11.5	27.2	5.5	10.7	20.5	4.6
8	10.1	48.9	7.6	7.9	21.8	3.8	10.1	56.6	3.1	11.5	16.8	2.0
MEAN ± SD	6.5 +- 3	44.7 +- 5.2	8.0 +- 2.7	9.3 +- 4.1	27.5 +- 5.9	4.7 +- 1.3	7.5 +- 2.3	45.1 +- 9.7	7.2 +- 3.6	8.4 +- 2.7	30.2 +- 2.7	4.0 +- 1.0

TABLE 17A EFFECT OF SMOKING 6 NORMAL CIGARETTES ON F.F.A. PATTERNS AS MEASURED BY GAS LIQUID CHROMATOGRAPHY

F.F.A. PATTERNS

SUBJECT	BEFORE SMOKING						AFTER SMOKING					
	C14	16	16.1	18	18.1	18.2	C14	16	16.1	18	18.1	18.2
1	13.6	49.6	7.6	4.6	24.5	-	6.4	51.0	5.0	4.7	32.7	-
2	9.6	56.2	5.6	3.9	20.9	3.5	10.3	46.4	8.6	8.3	21.4	4.6
3	4.2	52.2	7.7	6.0	25.4	4.2	5.7	47.4	9.0	1.6	30.9	5.1
4	5.9	47.7	8.9	-	31.4	5.9	3.3	44.1	9.8	2.8	35.5	4.4
5	6.4	43.1	13.4	2.2	28.7	5.9	4.5	42.2	16.2	1.8	28.9	6.1
6	7.3	48.5	4.8	12.9	22.9	3.5	5.4	60.7	4.5	4.5	21.5	3.3
7	8.9	53.7	4.9	10.6	17.5	6.1	11.5	47.2	5.5	10.6	20.5	4.6
8	9.8	57.3	6.1	4.0	19.6	3.2	9.0	47.0	8.5	3.6	26.2	5.7
MEAN +- SD	8.2 +- 2.9	51.0 +- 4.7	7.3 +- 2.8	6.3 +- 3.9	23.6 +- 4.6	4.6 +- 1.3	7.0 +- 2.9	48.3 +- 5.6	8.4 +- 3.7	4.7 +- 3.7	27.1 +- 5.7	4.8 +- 1.0

TABLE 17B EFFECT OF SMOKING 6 NORMAL CIGARETTES ON TRIGLYCERIDE FATTY ACID PATTERNS AS MEASURED BY GAS LIQUID CHROMATOGRAPHY

TRIGLYCERIDE FATTY ACID PATTERNS

CHAPTER TWELVE

EFFECT OF GLYCEROL INFUSION ON FFA LEVELS

This chapter describes studies undertaken to measure FFA and triglyceride values following glycerol infusion. When the Dole method was used for FFA measurement an unexpected rise following glycerol was found. The mechanisms involved in this finding are discussed in a later chapter.

STUDY A TO ASSESS THE EFFECT OF INFUSION OF 50 ML NORMAL SALINE ON LEVELS OF FFA AND GLYCEROL

Six subjects were studied. All were male, age range 30-56 years, mean 46 years. Their diagnoses were idiopathic hypertension (4) and mitral valve disease (2). After an overnight fast an intravenous cannula was inserted as before. A basal sample was taken and 50 ml of normal saline was infused slowly over 5 minutes. Venous samples were taken at 10 minute intervals (timed from the mid-point of the injection) for 60 minutes.

The results (Table 18) show that there was no change in levels of either FFA or glycerol suggesting that a satisfactory steady state had been established.

STUDY B TO ASSESS THE EFFECT OF INFUSION OF 50 ML 10% GLYCEROL ON LEVELS OF PLASMA FFA AND GLYCEROL

Thirteen subjects were studied. All were male, age range 30-60 years, mean 49 years. Their diagnoses were systemic

hypertension (4), mitral valve disease (2), angina pectoris due to CHD (3) and PVD (4). After an overnight fast and the introduction of a venous cannula, 50 ml of 10% glycerol were infused over 5 minutes. A sample was taken at 5 and 10 minutes and then at 10 minute intervals for 40 minutes. Initial studies showed a rise in FFA values after glycerol infusion when the Dole method was used. Accordingly, in the current investigation both Dole and Boehringer methods were used to measure FFA values.

The results (Table 19 and Figure 13) show that the peak value for glycerol is found at 5 minutes and that values then fall exponentially. Free fatty acids as measured by the Dole method rise with the maximum values being found 5-10 minutes after infusion whereas a fall occurs when the Boehringer method is used. The latter would be the expected response since glycerol is largely metabolised to glucose which in turn is known to depress FFA levels.

It was felt likely that this unexpected result was technical and perhaps associated with glycerol. However, the effect of smoking 2 normal cigarettes on lipolysis was rechecked using 3 methods of FFA measurement, (Dole; Trout, Ester and Friedberg and Boehringer). Nine subjects were studied and the results are shown in Table 20. All methods used show an increase in FFA levels following cigarette smoking with the response being similar for each method. (See chapter 14)

TABLE 18 EFFECT OF SALINE INFUSION ON FFA LEVELS (Mean ± SD)

Time (min)	FFA (Dole) ueq/l	Free Glycerol mg/100 ml
0	969 ± 251	1.1 ± .2
10	960 ± 245	1.2 ± .2
20	956 ± 244	1.1 ± .2
30	962 ± 244	1.1 ± .2
40	980 ± 231	1.2 ± .2
50	969 ± 230	1.2 ± .2
60	959 ± 209	1.2 ± .2

TABLE 19 EFFECT OF GLYCEROL INFUSION ON FFA AND GLYCEROL LEVELS (Mean ± SD)

Time (min)	FFA (Dole) ueq/l	FFA (Boehringer) ueq/l	Free Glycerol mg/100 ml
0	634 ± 192	615 ± 171	1.5 ± .6
5	809 ± 211	551 ± 212	8.8 ± 3.6
10	832 ± 225	534 ± 223	6.4 ± 3.4
20	796 ± 195	559 ± 238	4.1 ± 2.4
30	726 ± 214	552 ± 207	2.7 ± 1.7
40	681 ± 201	546 ± 176	2.1 ± 1.4

TABLE 20 EFFECT OF SMOKING 2 NORMAL CIGARETTES ON FFA LEVELS AS MEASURED BY METHODS OF (a) DOLE (b) TROUT, ESTES AND FRIEDBERG AND (c) BOEHRINGER (Mean ± SD)

Method	FFA ueq/l	15	30	45	60
Dole	701 ± 198	761 ± 174	781 ± 200	787 ± 210	733 ± 203
Trout, Estes, Friedberg	693 ± 199	747 ± 213	789 ± 224	773 ± 221	713 ± 193
Boehringer	709 ± 220	782 ± 221	818 ± 242	801 ± 239	738 ± 194

Effect of infusion of Glycerol on levels of FFA as measured by Dole and Boehringer.

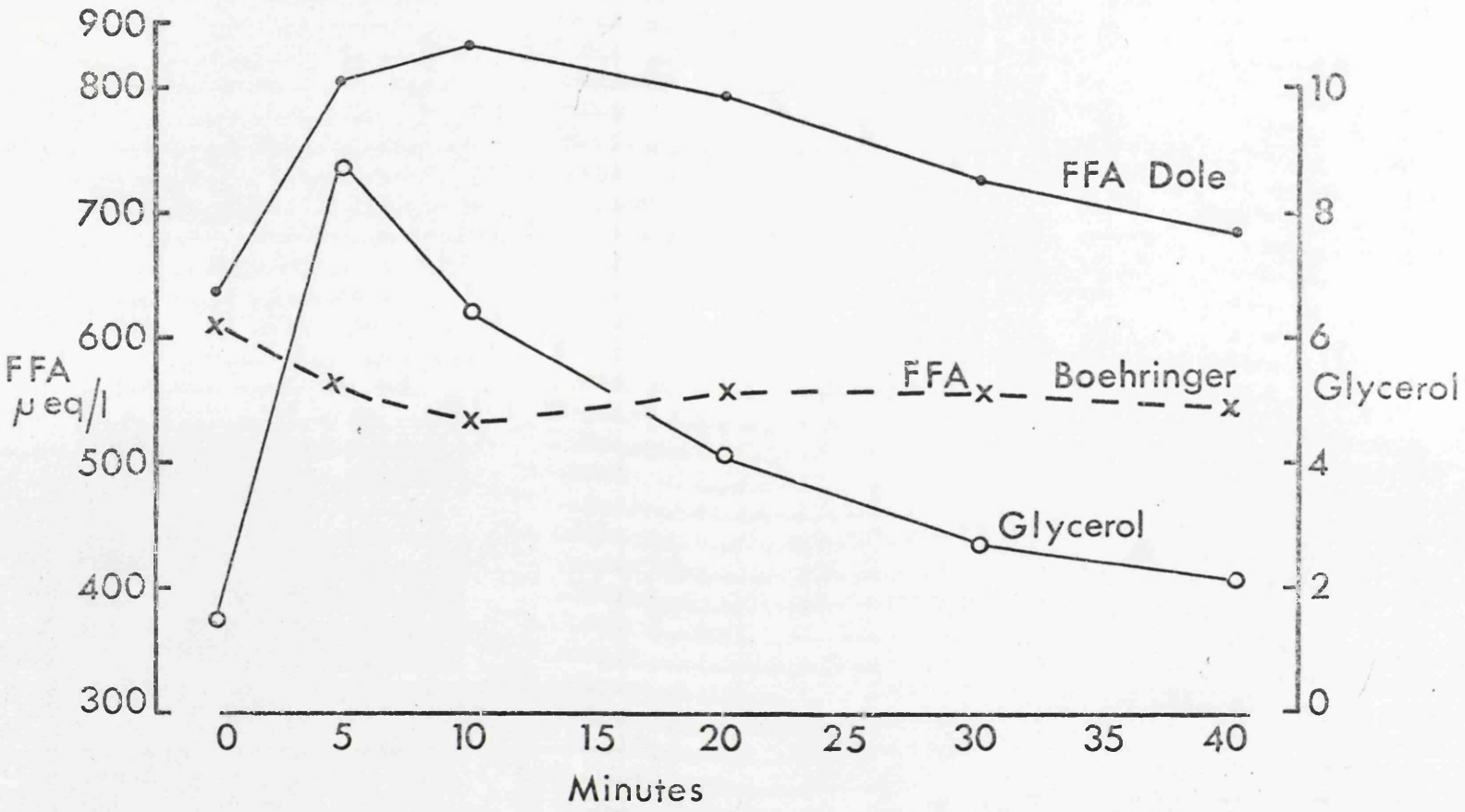


FIGURE 13

CHAPTER THIRTEEN

RATE OF GLYCEROL REMOVAL AND THE EFFECT OF CIGARETTE SMOKING

Free fatty acid and glycerol levels in the blood increase as a consequence of lipolysis. The previous studies described showed that ~~risen~~ raised levels of FFA found after smoking were associated with increased ketone body formation. It was decided to investigate the disappearance rate of glycerol from the plasma and whether or not this was influenced by cigarette smoking.

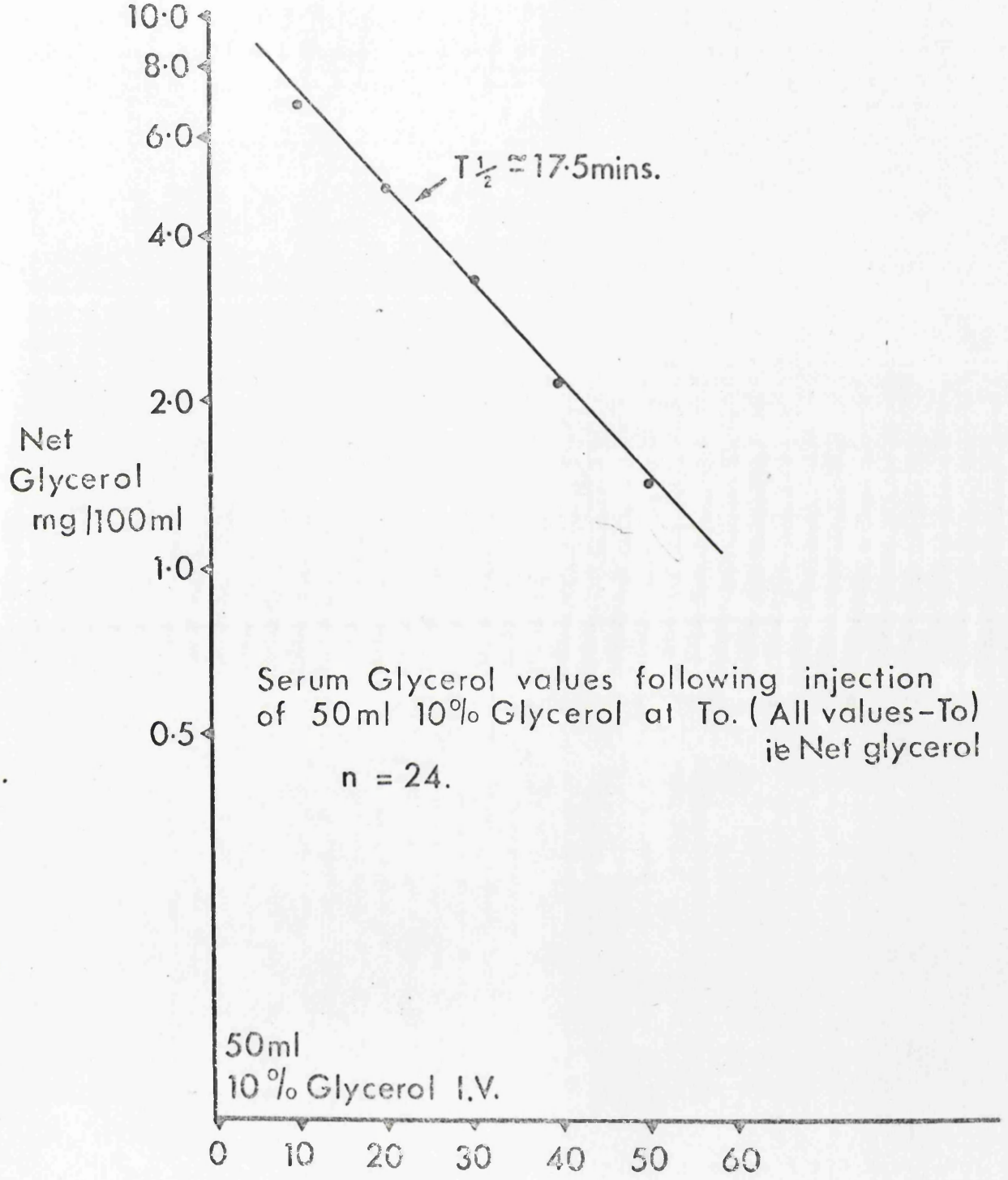
Tibbling (1969)[122] and Senior and Loridan (1968)[112] suggested that the rate of removal of intravenously administered glycerol could be expressed as a single exponential. This was also found in the present study. Figure 14 is composed of data obtained from 24 subjects. In each 50 ml of glycerol were infused after obtaining a basal sample with subsequent serial samples being withdrawn. Net glycerol values were obtained by subtracting the basal value from that found at each time interval after glycerol infusion. Mean values were plotted on semi-logarithmic paper and the line of best fit drawn. Individual glycerol T½ values have also been plotted against fasting cholesterol and triglyceride values (Figures 15 and 16). Little relationship was found between them. Mean fasting glycerol values and mean rate of glycerol removal were also obtained for

these subjects whose fatty triglyceride values were above and below 125 mg/100 ml. There was again no definite relationship between them (Table 21).

The effect of cigarette smoking on the rate of removal of glycerol from serum was studied before and during cigarette smoking in 7 male subjects (age range 34-48 years, mean 42 years). All were suffering from PVD. Studies were done on consecutive days after an overnight fast and then insertion of a venous cannula. On one day 50 ml of 10% glycerol were infused and serial venous samples obtained as before. On the other day (which could be either day 1 or day 2) the glycerol was again infused with each subject then smoking 1 normal nicotine content cigarette every 20 minutes for 1 hour.

Results are shown in Table 22. A wide range of values for glycerol T½ was found but there was no change in the rate of removal as a consequence of cigarette smoking.

FIGURE 14


10·0
8·0
6·0
4·0
2·0
1·0
0·5
Net
Glycerol
mg/100ml
T½ ≃ 17·5mins.
Serum Glycerol values following injection
of 50ml 10% Glycerol at To. (All values-To)
ie Net glycerol
n = 24.
50ml
10% Glycerol I.V.
0
10
20
30
40
50
60

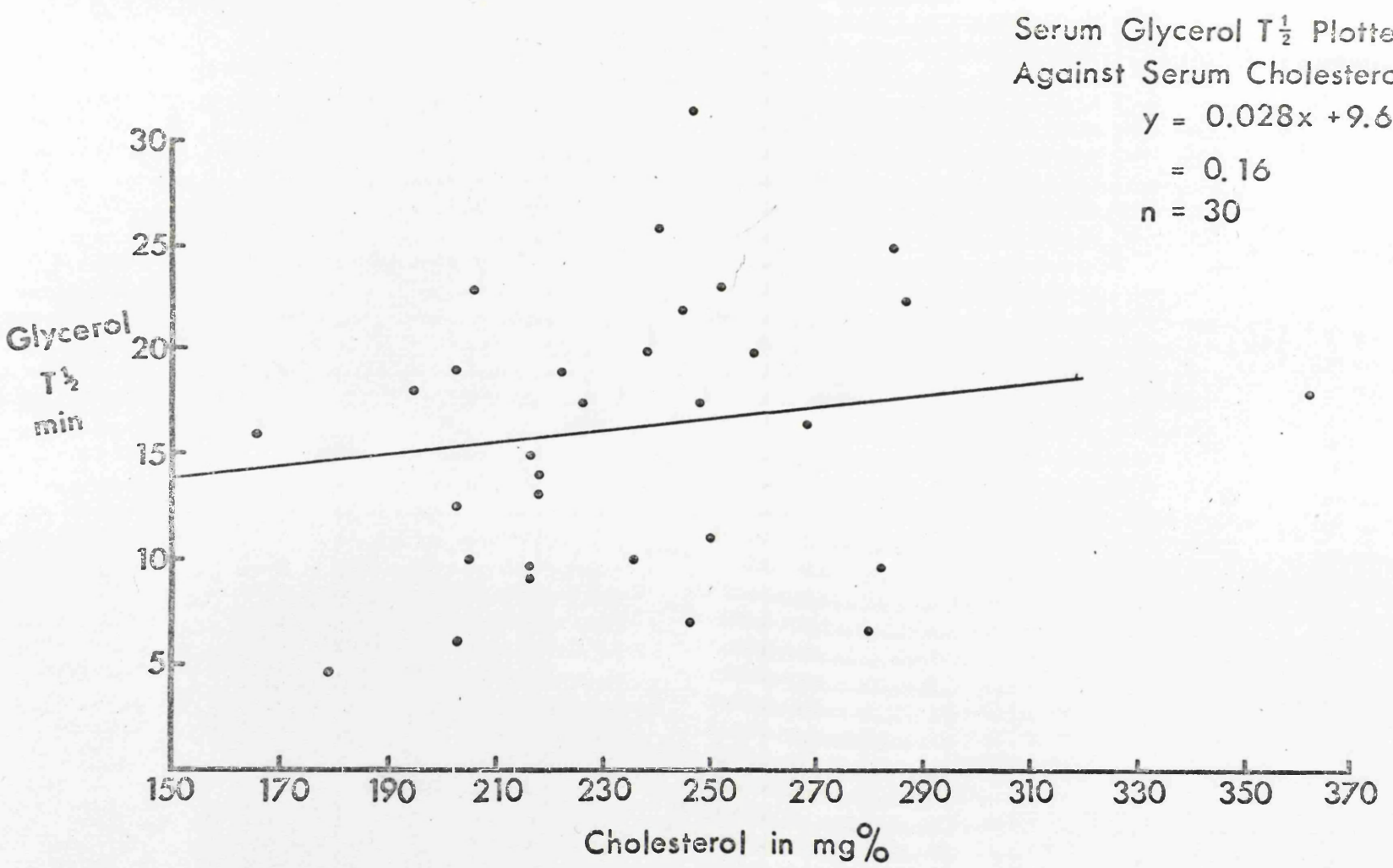

Serum Glycerol T½ Plotted
Against Serum Cholesterol
y = 0.028x +9.6
= 0.16
n = 30
Glycerol
T½
min
30
25
20
15
10
5
150
170
190
210
230
250
270
290
310
330
350
370
Cholesterol in mg%

FIGURE 15

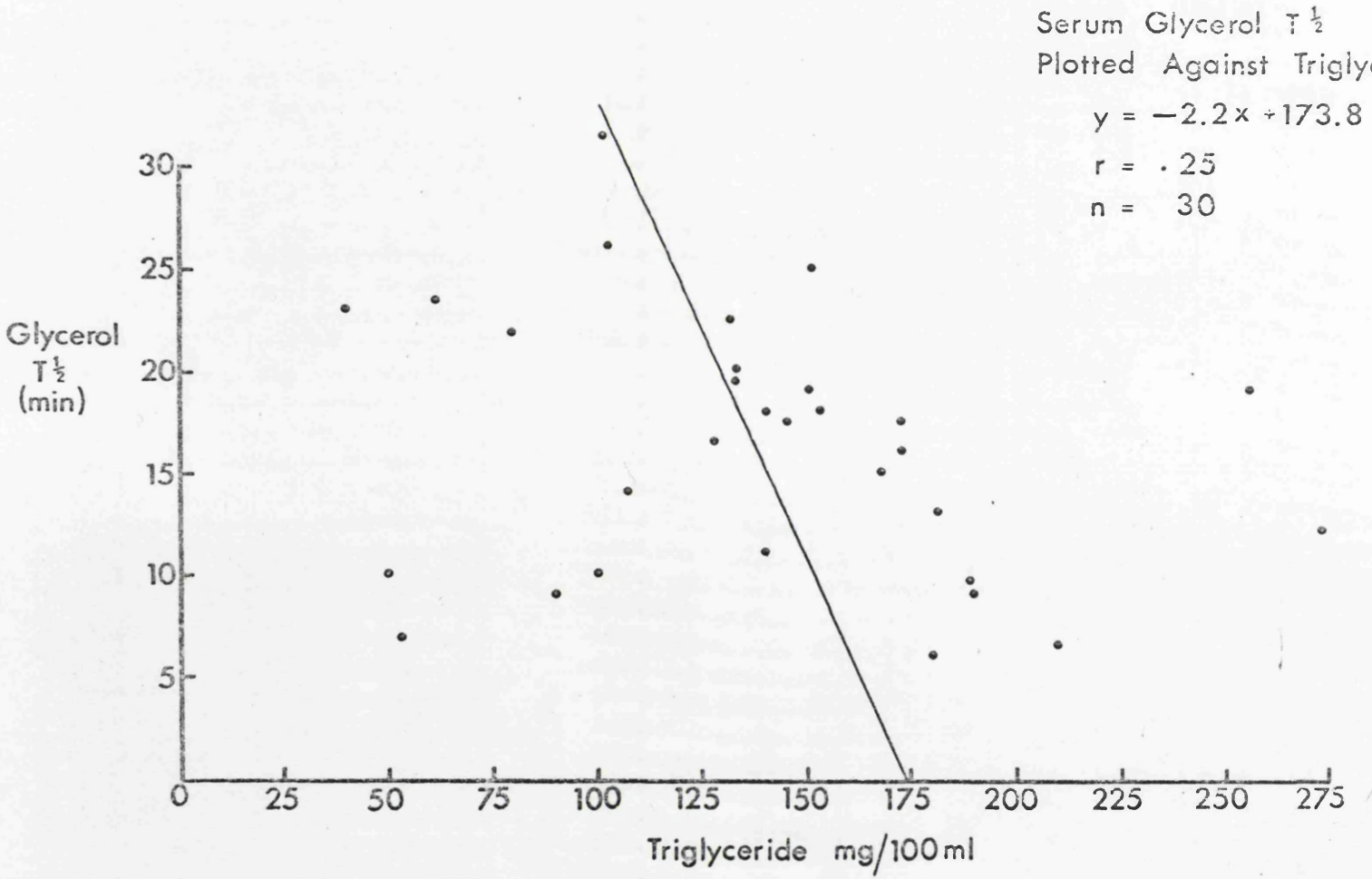

Serum Glycerol T ½
Plotted Against Triglyceride
y = −2.2x + 173.8
r = .25
n = 30
Glycerol T½ (min)
30
25
20
15
10
5
0
25
50
75
100
125
150
175
200
225
250
275
Triglyceride mg/100 ml

FIGURE 16

TABLE 21 RATE OF REMOVAL OF GLYCEROL WHEN FASTING TRIGLYCERIDE ABOVE OR BELOW 125 MG/100 ML

Group	No.	Age	Fasting TGL	Glycerol (mg/100 ml) TO	Glycerol T½ (min)
Fasting TGL 125 mg/100 ml	11	46 ± 10	71 ± 23	1.14 ± .45	15.5 ± 6.8
Fasting TGL 125 mg/100 ml	17	48 ± 8	173 ± 42	1.33 ± .74	14.9 ± 5.1

TABLE 22 EFFECT OF CIGARETTE SMOKING ON RATE OF REMOVAL OF GLYCEROL

Subject	Glycerol T½ (min)	
	Before Smoking	During Smoking
1	13.0	15.0
2	15.5	19.5
3	26.0	29.5
4	9.0	9.0
5	31.5	25.0
6	23.5	23.0
7	20.0	17.5
Mean ± SD	19.7 ± 7.8	19.5 ± 7.2

CHAPTER FOURTEEN

THE EFFECT OF INTRAVENOUS INFUSION OF GLYCEROL ON THE VALUES OBTAINED BY DIFFERENT ANALYTICAL PROCEDURES FOR THE PLASMA CONCENTRATIONS OF UNESTERIFIED FATTY ACIDS

INTRODUCTION

The method devised by Dole (1956)[25] for determining the concentration of unesterified fatty acids is known to be subject to certain errors (Gordon, Cherkes and Gates 1957;[39] Trout, Estes and Friedberg 1960).[124] According to Trout et al (1960) the lactic acid and phospholipids present in normal fasting plasma can interfere with the Dole (1956)[25] method to the extent that a positive error of about 5.5% is introduced into the values obtained for plasma unesterified fatty acid levels; this error increased as the plasma lactic acid increased with exercise, psychological stress or diabetes. In a study of the metabolism of glycerol in normal subjects and in patients with myocardial infarction or peripheral vascular disease, it was discovered that in plasma samples obtained after the intravenous infusion of glycerol, a positive error of up to 200% was introduced into the values for the concentrations of unesterified fatty acids determined by the Dole (1956)[25] procedure. An investigation of this very large error is now reported.

METHODS USED IN THE INVESTIGATION OF RAISED FFA LEVELS AFTER GLYCEROL INFUSION

FREE FATTY ACIDS

In view of the disagreement between the results obtained by the different methods, these will be described in some detail.

TITRIMETRIC METHOD

The method of Dole (1956)[25] was one of the two titrimetric procedures used in the investigation.

> 1.0 ml of plasma was shaken vigorously with 5.0 ml of isopropyl alcohol-heptane-NH_2SO_4 (40:10:1, by vol) and then allowed to stand for 10 min. This one phase system was then transformed into a two phase system by the addition of 3.0 ml of water and a further 2.0 heptane. 3.0 ml of the upper phase of heptane, containing the plasma unesterified fatty acid fraction, was added to 1.0 ml of 0.01% (w/v) thymol blue in ethanol-water (90:10, v/v) and titrated with 0.018 NaOH while a stream of N_2 was passed through the mixture.

As pointed out by Dole (1956)[25], this method cannot be regarded as being completely specific for unesterified fatty acids. Any acidic component of the plasma that passes into the heptane phase will be determined as "unesterified fatty acids". However,

Dole (1956)[25] was able to show that the addition of a-ketoglutaric, β-hydroxybutyric, indole acetic, succinic, glycerophosphoric, citric, malic, ascorbic or pyruvic acids to plasma resulted in negligible increases in the titratable acidity of the heptane phase. Moreover, when plasma was ultrafiltered through a collodion membrane, and the filtrate submitted to the extraction procedure, only about 5% of the original titratable acidity was found in the heptane phase. In addition, plasma was fractionated in a preparative ultracentrifuge; titratable acidity was found only in the heptane phase derived from the albumin fraction. On the basis of this evidence, Dole (1956)[25] concluded that the unesterified fatty acid fraction in the plasma represented by far the major proportion of the acidic material that passed into the heptane phase during the extraction procedure.

MODIFIED TITRIMETRIC METHOD

Mainly because of reports (Gordon 1957;[39] Fredrickson and Gordon 1958)[35] that elevated levels of plasma lactate interfered with the determination of the concentration of plasma unesterified fatty acids by the method of Dole (1956),[25] this procedure was modified by Trout, Estes and Friedberg (1960).[124] The method of Trout et al (1960) was the second of the two titrimetric methods used.

2.0 ml of plasma was shaken vigorously with 10.0 ml isopropyl alcohol-heptane-NH_2SO_4 (40:10:1 by vol) and then allowed to stand for 10 min. 6.0 ml heptane and 4.0 ml water

were added and the mixture was again shaken and then allowed to stand until complete separation of the two phases occurred. An aliquot (4.0 ml) of the upper heptane layer was shaken vigorously with an equal volume of 0.05% H_2SO_4 (v/v) and the mixture was then centrifuged at 300 x g for 5 min. 3.0 ml of the washed heptane phase and 1.0 ml of 0.01% thymol blue (w/v) in ethanol-water (90:10 v/v) were titrated with 0.018N NaOH in an atmosphere of N_2.

Trout et al (1961)[124] presented evidence that the introduction of the step involving the washing of the heptane layer with 0.05% H_2SO_4 reduced the interference from plasma lactate and plasma phospholipids.

The concentrations of lactic acid were determined in two samples of plasma by the method of Barker and Summerson (1941).[10] Portions of each of these plasma samples were then extracted either by Dole (1956)[25] procedure or by the Trout et al (1960)[124] procedure and the concentrations of lactic acid determined in the unwashed or washed heptane phases. Measured volumes of the heptane phases were evaporated under a stream of air at room temperature and the residue taken up in water, treated with $Ba(OH)_2$ and $ZnSO_4$, and filtered. The filtrate was analysed for lactic acid by the method of Barker and Summerson (1941).[10] About 2.0% of the total plasma lactic acid was found in the unwashed heptane phase, whereas only about 0.35% was found in the washed heptane phase.

Total plasma lipids were separated into phospholipids and non-phospholipids by chromatography on a column of silic acid (Hirsch and Ahrens 1958).[49] The phospholipid fraction was extracted by the Dole (1956)[25] procedure and the unwashed heptane phase found to have appreciable titratable acidity; this was markedly reduced when the heptane phase was washed with 0.05% H_2SO_4.

Two large samples of plasma were extracted by the Dole (1956)[25] procedure and the titratable acidity determined in portions of the unwashed and washed heptane phases. The mean values for the titratable acidities (umole/ml of original plasma) for the unwashed and washed heptane phases were 270 and 223 respectively. Thus, washing with 0.05% H_2SO_4 considerably reduced the titratable acidity of the heptane phase. Other portions of the unwashed and washed heptane phases were taken to dryness at 60°C under reduced pressure; this procedure removed the heptane as well as the lactic acid. The residues were taken up in heptane and the mean titratable acidities (umole/ml of original plasma) for the unwashed and washed heptane phases were 246 and 217 respectively. Thus the removal of lactic acid from both the unwashed and washed heptane phases were also taken to dryness at 60°C under reduced pressure in order to remove lactic acid. The residues were taken up in 0.5 ml of light petroleum (bp 30-50°C). Acetone was added and the phospholipids were precipitated during storage overnight at -20°C. The precipitated phospholipids were removed by

centrifugation and the solvent was removed from the supernatant layer. The residues were taken up in heptane and the mean titratable acidities (umole/ml of original plasma) for the unwashed and washed heptane phases were 224 and 216 respectively. From these results, Trout et al (1960)[124] concluded that in the original Dole (1956)[25] procedure, the titratable acidity of the heptane phase, although accounted for mainly by the plasma unesterified fatty acids is also influenced by plasma phospholipids and small amounts of plasma lactate that pass into the heptane phase. These contaminants were largely removed by washing the heptane phase with 0.05% H_2SO_4.

To investigate the effect of the washing procedure on the recovery of fatty acids, Trout et al (1960) added 1-^{14}C)-palmitic acid to plasma samples. The labelled palmitic acid was extracted quantitatively into the heptane phase during the Dole (1956)[25] extraction procedure. When the heptane phase was washed with 0.05% H_2SO_4, 99.7% of the labelled acid remained in the heptane phase and 0.3% was removed in the washing solution.

COLORIMETRIC METHOD

The method used was that described in the Biochemica Test Combination Cat. No. 15997 TFAB (Boehringer Corporation, London Ltd) and based on the procedures described by Duncombe (1964)[28] and Itaya and Ui (1965).[53]

0.2 ml of plasma was added to 5.0 ml of chloroform and 1.0 ml of 0.27M cU $(NO_3)_2$ in 0.45M triethanolamine

buffer, pH 7.8. The mixture was shaken vigorously for 10 min and then centrifuged for 5 min at 300 x g. The upper aqueous layer and any precipitated proteins were removed from the chloroform layer which contained the copper salts of the long-chain unesterified fatty acids originally present in the plasma. 2.0 ml of the chloroform layer were then mixed with 0.2 ml of 9mM diethyldithiocarbamate in butanol, a reagent used for the colorimetric microdetermination of copper. After 10 min the optical density was measured at 440 nm.

According to Duncombe (1964)[28] and Itaya and Ui (1965)[53], a high degree of specificity for the unesterified fatty acid fraction of plasma is obtained with this method.

GAS-LIQUID CHROMATOGRAPHIC METHOD

The method used was that described by Christie, Noble and Moore (1970)[23] and although more laborious, it may be regarded as being more specific for the unesterified fatty acids of plasma than any of the other three methods.

Total lipids were extracted from plasma samples by the method of Nelson and Freeman (1959)[90]. To a portion of the lipid extract a known amount of methyl heptadecanoate was added as an internal standard and the mixture was refluxed for 2 h with methanol containing 5% anhydrous

hydrogen chloride. Gas-liquid chromatographic analysis of the resulting methyl esters of the total plasma fatty acids was carried out on columns of 15% (w/v) polyethylene glycol adipate on Chromosorb W (100-120 mesh, acid washed and silanised: Phase Separations Ltd, Rock Ferry, Cheshire) in a Pye 104 chromatograph. From these results the total concentration of each fatty acid in the plasma could be calculated. A further portion of the total plasma lipid extract was chromatographed on Kieselgel G plates with a solvent system of hexane-diethyl ether-formic acid (80:20:2, by vol). The component lipid classes were detected by spraying with 0.1% (w/v) 2:4; dichlorofluorescein in methanol and identified by comparison with standard mixtures. Bands were scraped off on to small columns of silicic acid from which the cholesteryl esters, triglycerides and unesterified fatty acids were eluted with diethyl ether, and the phospholipids with methanol containing 5% anhydrous hydrogen chloride. A known amount of methyl heptadecanoate was added as internal standard to each fraction. The cholesteryl esters and triglycerides were dissolved in a small amount of benzene and trans-esterified with 0.5M sodium methoxide in dry methanol. The 5% (w/v) boron trifluoride in methanol, and the phospholipids were trans-esterified with the methanolic hydrogen chloride used to elute from the

Kieselgel G. From the gas-liquid chromatographic analyses of the methyl esters obtained from each fraction, it was possible to calculate the percentage distribution of the fatty acids between the various lipid classes, and hence the plasma concentration of each lipid class, including the unesterified fatty acids.

STUDIES 1 and 2 — EFFECT OF INTRAVENOUS GLYCEROL INFUSION ON THE PLASMA CONCENTRATIONS OF UNESTERIFIED FATTY ACIDS AS MEASURED BY THE DOLE (1956) METHOD

Fifteen male subjects were each given an intravenous infusion of 50 ml of 10% glycerol in isotonic saline after an overnight fast. Blood samples were taken immediately before and 20 and 50 min after the glycerol infusion. In a more detailed investigation, 4 male subjects were each given a similar infusion of glycerol, but blood samples were taken before and 10, 20, 30, 40 and 50 min after the infusion. The concentrations of unesterified fatty acids were determined in the plasma samples by the method of Dole (1956)[25]. The mean results for Study 1 and the individual results for Study 2 are given in Tables 23 and 24 respectively. The values obtained for the concentration of unesterified fatty acids in the plasma of subjects after an overnight fast are similar to those reported by Dole (1956)[25] and Gordon, Cherkes and Gates (1957)[39]. In both studies apparent intravenous infusion of glycerol resulted in a pronounced increase in the plasma concentrations of unesterified fatty acids. This effect was observed in the individual results obtained from all of the subjects in Study 1. It appeared that the plasma unesterified fatty acids reached maximum concentrations 10 min after the glycerol infusion; thereafter, the concentration of unesterified fatty acids gradually decreased until at 50 min, the values were similar to those observed immediately before the glycerol infusion.

Table 23, Study 1 Effect of intravenous glycerol infusion on the plasma concentration of unesterified fatty acids (ueq/l) as determined by the method of Dole (1956)
(15 subjects; mean values with their standard errors)

Time after glycerol infusion (min)		
0	20	50
950 ±51.5	1195 ±62.3	1012 ±61.5

Table 24, Study 2 Effect of intravenous glycerol infusion on the plasma concentration of unesterified fatty acids (ueq/l) as determined by the method of Dole (1956) (4 subjects; individual values and their means)

	Time after glycerol infusion (min)					
Subject	0	10	20	30	40	50
A	1295	1638	1524	1406	1365	1343
B	685	932	903	821	714	659
C	780	1012	960	900	830	795
D	159	344	318	263	211	185
Mean	730	982	926	848	780	745

STUDY 3 EFFECT OF INTRAVENOUS INFUSION OF ISOTONIC SALINE ON THE PLASMA CONCENTRATION OF UNESTERIFIED FATTY ACIDS AS DETERMINED BY THE DOLE (1956) METHOD

Before further investigation of the unexpected findings of Studies 1 and 2, it was necessary to ascertain that the infusion of normal saline was without effect on the plasma unesterified fatty acid levels. In Study 3, 6 subjects were each given an intravenous infusion of 50 ml of isotonic saline after an overnight fast. Blood samples were taken immediately before and 10, 30, 40 and 60 min after the infusion. The concentrations of unesterified fatty acids were determined in the plasma samples by the method of Dole (1956).[25] The results given in Table 25 show that the plasma levels of unesterified fatty acids were unaffected by the saline infusion.

STUDY 4 EFFECT OF INTRAVENOUS GLYCEROL INFUSION ON THE PLASMA CONCENTRATION OF UNESTERIFIED FATTY ACIDS AS INVESTIGATED BY QUALITATIVE THIN-LAYER CHROMATOGRAPHY

The results of Studies 1 - 3 were particularly surprising in view of the fact that glycerol is known to contribute to gluconeogenesis in man (eg Cahil et al 1966;[16] Exton 1972).[33] Moreover, Senior and Loridan (1968)[112] have reported that an intravenous infusion of glycerol resulted in an increase in serum glucose levels and a decrease in serum unesterified fatty

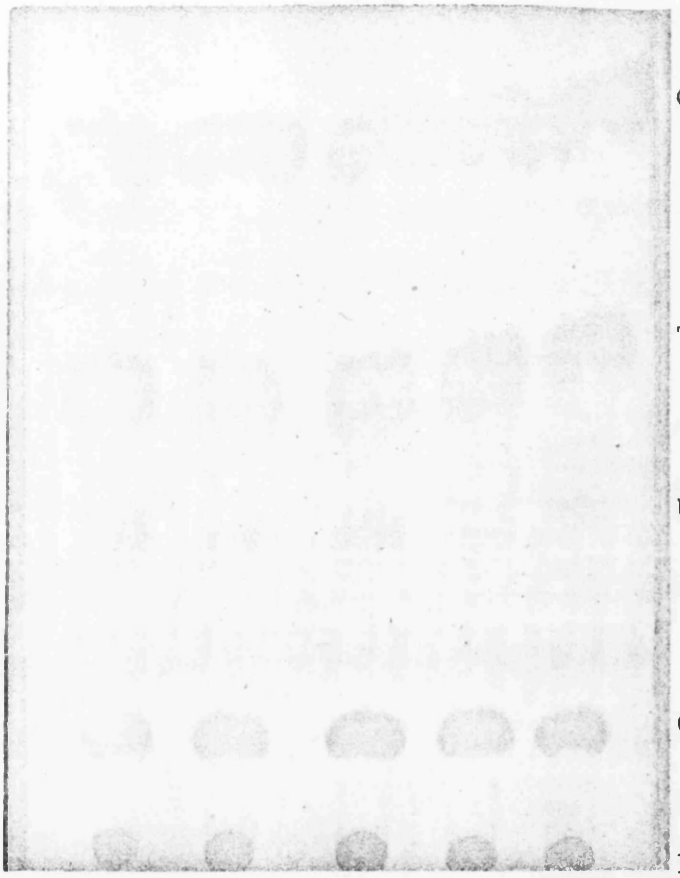
acid levels. It seemed possible, therefore, that the values given in Tables 23 and 24 did not represent a true assessment of the actual concentratians of plasma unesterified fatty acids.

Three subjects were each given an intravenous infusion of glycerol as previously described. Blood samples were taken before and at 5, 10, 30 and 30 minutes after the glycerol infusion. The concentration of unesterified fatty acids was determined on a portion of each plasma sample by the method of Dole (1956)[25] and the results (not given in detail) were found to be very similar to those in Tables above, ie the glycerol infusion resulted in an increase in the plasma concentration of unesterified fatty acids. The total lipids were extracted from another portion of each plasma sample by the method of Nelson and Freeman (1959).[90] Portions of the lipid extracts, equivalent to the same volume of plasma, were applied to thin-layer plates of Kieselgel G. The plates were developed with a solvent system of hexane-diethyl ether-formic acid (80:20:1, by vol). After development, the chromoplates were sprayed with 50% (v/v) sulphuric acid and then heated at 100^{0} for 1 hour. The results for all three subjects were similar and a photograph of a typical thin-layer chromatogram is given in Figure 17. From a qualitative assessment of each chromatogram it was clear that the glycerol infusion resulted in a marked decrease in the concentration of unesterified fatty acids in the plasma and in the total plasma lipids.

Table 25, Study 3 Effect of intravenous infusion of isotonic saline on the plasma concentration of unesterified fatty acids (μeq/l) as determined by the method of Dole (1956) (6 subjects; mean values with their standard deviations)

Time after glycerol infusion (min)					
0	10	20	30	40	60
969 ±251	960 ±245	956 ±244	962 ± 244	980 ±231	959 ±209

FIGURE 17



Photograph of a thin-layer chromatogram of the plasma lipids of a subject given an intravenous infusion of glycerol.

STUDY 5 COMPARISON OF PLASMA UNESTERIFIED FATTY ACID CONCENTRATIONS DETERMINED BY THE DOLE METHOD WITH THOSE DETERMINED BY A COLORIMETRIC METHOD AND BY A GAS-LIQUID CHROMATOGRAPHIC METHOD

Since the qualitative results obtained from Study 4 were in complete disagreement with the results of Studies 1 and 2 an investigation, similar to Study 4, was undertaken but each plasma sample was analysed for unesterified fatty acid concentrations by three methods based on entirely different chemical principles ie, a titrimetric procedure (Dole 1956)[25], a colorimetric procedure (Duncombe 1964)[28] and a quantitative gas-liquid chromatographic procedure (Christie et al 1970)[23].

Four subjects were each given an intravenous infusion of glycerol as described previously. Blood samples were taken before and 5, 10, 20, 30 and 40 minutes after the glycerol infusion and the concentration of unesterified fatty acids determined in each sample by the three separate methods. From the results given in Table 26, it is evident that the pattern of results obtained by the titrimetric (Dole 1956)[25] method was very similar to that obtained in Study 1. However, the results obtained both by the colorimetric (Duncombe 1964)[28] and gas-liquid chromatographic (Christie et al 1970)[23] indicated that the glycerol infusion resulted in a pronounced decrease in the concentration of plasma unesterified fatty acids; minimum values were observed 20 min after the glycerol infusion, but even after 40 min, plasma unesterified fatty acid levels were only about 57% of the level observed immediately before the glycerol infusion. It should be noted that there was good

agreement between the results obtained from corresponding plasma samples by the colorimetric and gas-liquid chromatographic methods. It should also be noted that there was good agreement between the results obtained by all three methods when applied to the plasma samples obtained before the glycerol infusion.

STUDY 6 COMPARISON OF PLASMA UNESTERIFIED FATTY ACID CONCENTRATIONS DETERMINED BY THE DOLE METHOD WITH THOSE DETERMINED BY A COLORIMETRIC METHOD AND BY A MODIFIED DOLE METHOD

The results of Study 5 suggested that the intravenous infusion of glycerol resulted in the appearance in the plasma of some acidic component that was not a long-chain fatty acid; this unknown acidic component appeared to pass into the heptane phase where it was titrated as "unesterified fatty acids" in the original Dole (1956)[25] procedure. It was of interest, therefore, to determine whether this contaminant could be removed from the heptane phase by washing with 0.05% H_2SO_4 as in the modification of the Dole (1956)[25] method devised by Trout et al (1960).[124]

In Study 6, 4 subjects were each given an intravenous glycerol infusion. Blood samples were taken before and 10, 20, 30, 40 and 50 min after the glycerol infusion and the concentrations of unesterified fatty acids in each plasma sample determined by a titrimetric procedure (Dole 1956),[25] a modified titrimetric procedure (Trout et al 1960)[124] and a colorimetric procedure (Duncombe 1964).[28] The pattern of results obtained by the titrimetric (Dole, 1956)[25] method (Table 27) were similar to those

obtained previously (see Tables 22 and 24), and showed that the glycerol infusion resulted in a transient but pronounced increase in the concentration of plasma unesterified fatty acids. The results obtained by the colorimetric (Duncombe 1964)[23] method (Table 27) were also similar to those obtained previously (Table 26) and showed a marked decrease in the concentration of plasma unesterified fatty acids as a result of the glycerol infusion. When determined by the modified titrimetric (Trout et al 1960)[124] procedure, the concentration of unesterified fatty acids in the plasma was not changed appreciably by the glycerol infusion. Again, it should be noted that there was extremely good agreement between the values obtained by all three methods when applied to the plasma samples obtained before the glycerol infusion.

The findings of Study 6 again suggest that the glycerol infusion resulted in the appearance in the plasma of some unknown acidic component that passed into the heptane phase in the Dole (1956)[25] procedure. This contaminant appeared to be only partially removed by the 0.05% H_2SO_4 wash introduced into the procedure by Trout et al (1960).[124] Thus, in the plasma sample obtained 10 min after the glycerol infusion, the total titratable acidity in the heptane phase amounted to 1086 u equiv/l plasma. The titratable acidity in the heptane phase due to the contaminant thus amounted to 463 u equiv/l plasma; only 68% of this contaminant (amounting to 316 u equiv/l plasma) was removed from the heptane by the 0.05% H_2SO_4 wash.

Table 26, Study 5 Effect of intravenous infusion of glycerol on the plasma concentration of unesterified fatty acids (ueq/l) as determined by a titrimetric method (Dole 1956), a colorimetric method (Duncombe 1964) and a quantitative gas-liquid chromatographic method (Christie et al 1970). (4 subjects; mean values with their standard errors)

Time after glycerol infusion (min)	Method of Analysis		
	Titrimetric	Colorimetric	Gas-liquid chromatographic
0	682 ±105	698 ±120	666 ±136
5	922 ±84.6	444 ±75.6	422 ±87.4
10	999 ±77.0	339 ±77.5	374 ±95.8
20	908 ±55.0	337 ±90.3	337 ±92.2
30	768 ±80.9	347 ±95.8	350 ±86.1
40	707 ±91.1	396 ±108.5	391 ±87.5

Table 27, Study 6 Effect of intravenous infusion of glycerol on the plasma concentration of unesterified fatty acids (ueq/l) as determined by a titrimetric method (Dole 1956), a modified titrimetric method (Trout et al 1960) and a colorimetric method (Duncombe 1964)
(4 subjects; mean values with their standard errors)

Time after glycerol infusion (min)	Method of analysis		
	Titrimetric	Modified titrimetric	Colorimetric
0	797 ±75.2	783 ±76.1	781 ±75.1
10	1086 ±84.9	770 ±74.2	623 ±46.3
20	1028 ±59.4	753 ±60.5	530 ±54.8
30	935 ±65.4	761 ±62.9	584 ±51.4
40	860 ±86.8	763 ±68.1	616 ±64.6
50	798 ±75.4	762 ±72.5	658 ±74.5

Study 7 THE RELATIONSHIP BETWEEN THE CONCENTRATIONS OF UNESTERIFIED FATTY ACIDS, AS DETERMINED BY TITRIMETRIC AND COLORIMETRIC METHODS, AND FREE GLYCEROL IN THE PLASMA OF SUBJECTS GIVEN AN INTRAVENOUS INFUSION OF GLYCEROL

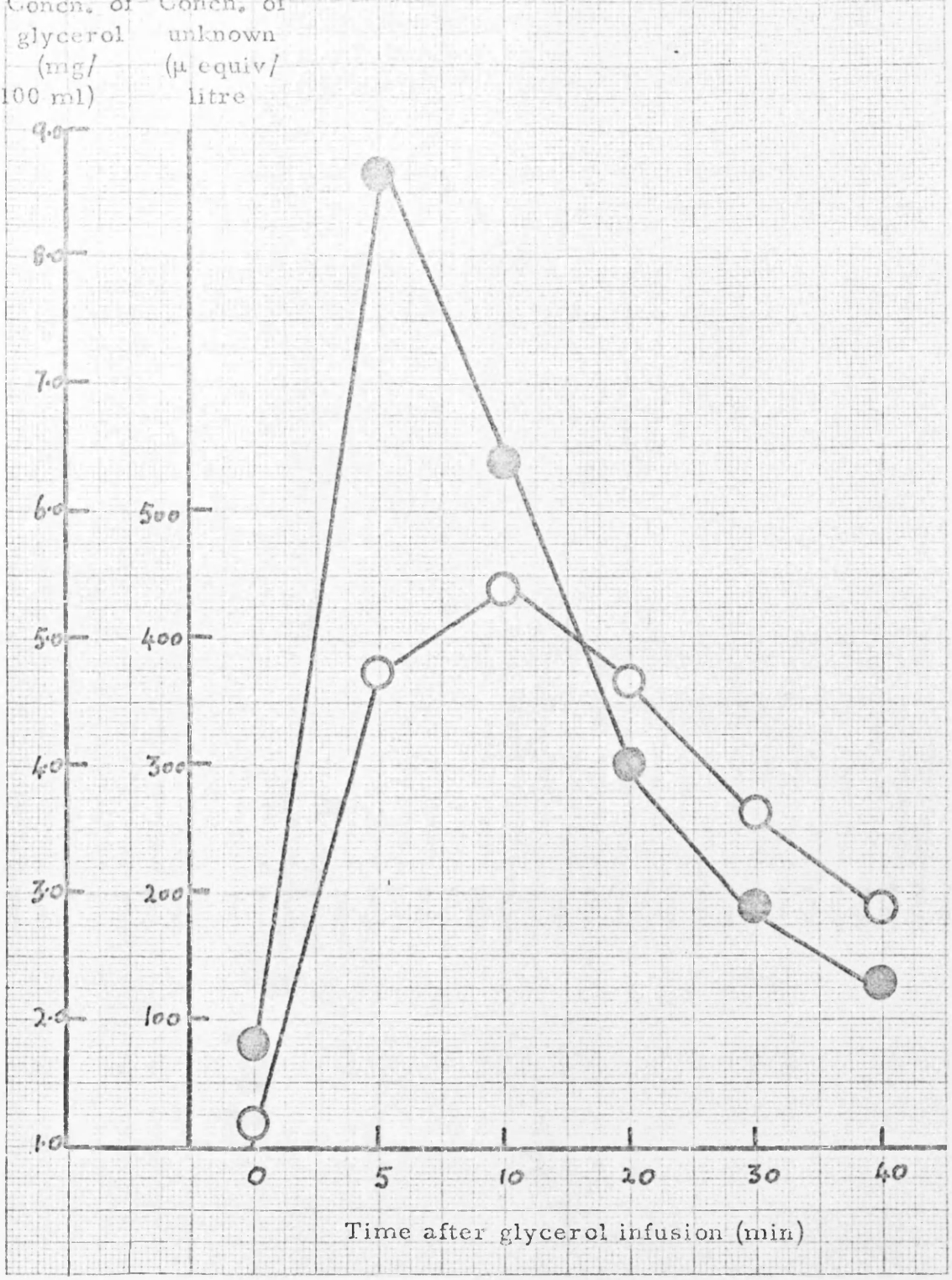
From the previous results it appeared that the unknown acidic component that interfered with the Dole[25] (1956) method of determining the concentration of unesterified fatty acids only appeared in the plasma after the glycerol infusion. It seemed possible that a study of the relationships between the subsequent concentrations of glycerol and the unknown acidic component might provide some hint as to the identity of the latter. Some estimate of the concentrations of the unknown component could be obtained simply by subtracting the values for the plasma concentrations of unesterified fatty acids obtained by the method of Duncombe[28] (1964) from those obtained by the method of Dole[25] (1956).

In Study 7, 10 subjects were each given an intravenous infusion of glycerol. Blood samples were taken before and 5, 10, 20, 30 and 40 minutes after the glycerol infusion and the plasma concentrations of free glycerol determined by the methpd described in the Biochemica Test Combination Cat. No. 15989 (Boehringer Corporation London Ltd) and based on the procedures of Eggstein[29] (1966) and Eggstein and Kreutz[30] (1966). Plasma unesterified fatty acid levels were determined by the titrimetric (Dole[25] 1956) method and the colorimetric (Duncombe[28] 1964) method.

The results for the plasma concentrations of unesterified fatty acids as determined by the two analytical procedures (Table 28, Figure 7) are similar to those obtained in Studies 5 and 6 (Tables 26 and 27). The concentration of free glycerol in the plasma samples obtained immediately before the glycerol infusion (Table 27) was similar to that reported by others (eg Eggstain 1966)[29] for subjects after an overnight fast. Maximum concentrations of free glycerol were observed in the plasma samples taken 5 minutes after the glycerol infusion: between 5 and 40 minutes there was a rapid decline in the free glycerol level in the plasma (Table 28). Maximum concentrations of the unknown acidic component were found in the plasma samples taken 10 minutes after the glycerol infusion (Table 28 and Figure 18). There appeared to be a product-precursor relationship between the concentration of free glycerol and the concentration of the unknown acidic component in the plasma. Therefore, it seemed unlikely that the unknown acidic component in the plasma was a contaminant in the glycerol solution used for intravenous infusion; had this been so, maximum concentrations of glycerol and the unknown component might have been expected to occur in the same plasma sample. Moreover, no titratable acidity was detected in the glycerol solutions used for infusion. The results of Study 7 suggest, therefore, that the unknown component was (a) a product of glycerol metabolism, or (b) a product of some metabolic process stimulated by the glycerol infusion.

Table 28, Study 7 Relationship between the concentrations of unesterified fatty acids (ueq/l), as determined by a titrimetric method (Dole 1956) and a colorimetric method (Duncombe 1964), and the concentration of free glycerol (mg/100 ml) in the plasma of subjects given an intravenous infusion of glycerol.
(10 subjects; mean values with their standard errors)

Concentration of unesterified fatty acids (ueq/l)		Concentration of free glycerol (mg/100 ml)
Titrimetric method	Colorimetric method	
674 ±37.5	658 ±52.3	1.79 ±0.59
907 ±46.8	533 ±57.6	8.69 ±1.34
928 ±52.8	485 ±52.2	6.41 ±1.24
824 ±45.4	494 ±52.9	4.00 ±0.86
770 ±54.3	506 ±51.2	2.88 ±0.61
707 ±53.2	523 ±52.0	2.28 ±0.50



Relationship between the concentrations of free glycerol (●) and the unknown acidic component (○) in the plasma of subjects given an intravenous infusion of glycerol. Values for the unknown acidic component were obtained by subtracting the plasma concentrations of unesterified fatty acids determined by the method of Duncombe (1964) from those determined by the method of Dole (1956).

STUDY 8 EFFECT OF INTRAVENOUS GLYCEROL INFUSION ON PLASMA LIPID COMPOSITION

According to Trout et al (1960)[124], phospholipids pass into the heptane phase when plasma is extracted by the original Dole (1956)[25] procedure, and these phospholipids contribute to the titratable acidity of the heptane phase. Titratable acidity due to contaminating phospholipids was apparently removed when the heptane phase was washed with 0.05% H_2SO_4 (Trout et al 1960)[124]. To investigate whether the intravenous infusion of glycerol resulted in increased plasma concentrations of acidic phsopholipids or of any other polar lipids, infusion experiments were done as before, but the plasma samples were submitted to a complete lipid analysis.

Three subjects were each given an intravenous infusion of glycerol. Blood samples were taken before and 5, 10, 20, 30 and 40 minutes after the glycerol infusion, and the concentrations of unesterified fatty acids were determined in a portion of each plasma sample by the method of Dole (1956)[25]. The total lipids were extracted from the remainder of each plasma sample by the method of Nelson and Freeman (1959)[90]. The concentrations of total plasma fatty acids and of each plasma lipid class were then determined by quantitative gas-liquid chromatography (Christie et al 1970)[23]. In the lipids extracted from the plasma samples obtained before and 10 minutes after the glycerol infusion the composition of the phospholipids was determined as described by Skipski, Peterson and Barclay (1964)[115] and Christie et al (1970)[23].

The pattern of results for the plasma concentrations of unesterified fatty acids determined by the Dole (1956)[25] method and by quantitative gas-liquid chromatography (Table 29) was similar to that observed in previous experiments (Tables above). During the first 10 minutes after the glycerol infusion there was an increase in the concentration of plasma unesterified fatty acids as measured by the titrimetric procedure and a decrease when the plasma level of unesterified fatty acids was determined by quantitative gas-liquid chromatography (Table 29). Although there was considerable variation between subjects, the results given in Table 29 show that the intravenous infusion of glycerol had little or no effect on the plasma concentration of total fatty acids, cholesteryl esters, triglycerides or total phospholipids. Examination of the fatty acid compositions of each plasma lipid fraction (Tables 30 to 33) again revealed considerable variation between subjects but virtually no change in fatty acid composition occurred as a result of glycerol infusion. More than 98% of the unesterified fatty acid fraction could be accounted for by palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids, and careful temperature programmed gas-liquid chromatographic analysis revealed that the glycerol infusion did not result in increases in the trace concentrations of unesterified short and medium chain fatty acids (C_2 to C_{14}) that are usually present in plasma.

The compositions of the plasma phospholipids in the three subjects immediately before and 10 minutes after the infusion of

glycerol are given in Table 34. The appearance of small concentrations of phosphatidic acid in the plasma of subject E, 10 minutes after the glycerol infusion was of particular interest, for this phospholipid could well contribute to the titratable acidity of the heptane extract in the Dole (1956)[25] procedure. From the results for the plasma samples obtained from subject E 10 minutes after the glycerol infusion, it may be calculated that the titratable acidity of the non-fatty acid contaminant of the heptane phase of the Dole (1956)[25] extract amounted to about 700 u equivalents/litre of plasma. Even if all of the phosphatidic acid in the plasma of subject E obtained 10 minutes after the glycerol infusion passed into the heptane phase during the Dole (1956)[25] extraction (a most unlikely situation, in view of the polarity of phosphatidic acid), it may also be calculated that this amount of phosphatidic acid could only account for about 10% of the titratable acidity due to the non-fatty contaminant in the heptane phase. Moreover, phosphatidic acid was not detected in the plasma of subjects F and G after glycerol infusion. In all three subjects, glycerol infusion resulted in a decrease in the proportion of phosphatidyl choline and an increase in the proportion of sphingomyelin in the plasma phospholipids (Table 34). The reason for this change in plasma phospholipid composition is as yet unclear but it is unlikely that such a change in the phosphatidyl choline:sphingomyelin ratio in the phospholipids extracted by the heptane could exert any appreciable influence on the titratable acidity of the heptane phase.

TABLE 29, STUDY 8 Effect of intravenous glycerol infusion on the plasma concentration of unesterified fatty acids (ueq/l) as determined by a titrimetric method (Dole 1956), and on the plasma concentrations of each lipid fraction (mg fatty acid/100 ml) as determined by a gas-liquid chromatographic (GL) method (Christie et al 1970).

(3 subjects; individual values)

Plasma lipid fraction	Subject	Time after glycerol infusion (min) 0	5	10	20	30	40
Unesterified fatty acids (ueq/l) by titrimetric method	E	710	940	962	870	807	
	F	820	1030	1147	999	881	810
	G	580	777	899	770	670	530
Total fatty acids (mg/100 ml) by GLC	E	286	275	271	275	268	
	F	325	290	306	294	299	293
	G	194	179	182	177	183	189
Unesterified fatty acids (mg/100 ml) by GLC	E	19.3	9.8	6.7	7.3	8.6	
	F	23.0	10.4	8.9	9.9	12.0	13.0
	G	16.5	6.0	3.5	3.7	7.3	9.3
Cholesteryl esters (mg fatty acid/100 ml) by GLC	E	78.2	85.3	79.6	83.5	74.1	
	F	93.2	91.0	93.9	89.9	96.3	90.6
	G	69.3	71.0	72.2	65.2	67.3	68.6
Triglycerides (mg fatty acids/100 ml) by GLC	E	74.3	75.2	73.4	70.8	70.4	
	F	89.3	83.0	56.7	79.2	83.7	85.6
	G	35.4	33.2	38.4	38.0	38.4	39.2
Phosphalipids (mg fatty acids/100 ml) by GLC	E	113.8	104.5	111.0	113.0	114.9	
	F	119.5	106.0	117.1	115.0	107.1	104.0
	G	72.7	68.9	67.6	70.1	69.8	71.5

TABLE 30, STUDY 8 Fatty acid compositions (wt. percentages of the totals) of the plasma unesterified fatty acids of subjects given an intravenous infusion of glycerol

	Time after glycerol infusion (min)					
	0	5	10	20	30	40
Subject E						
16:0	25.4	26.5	26.3	25.4	24.7	
16:1	4.8	5.8	5.4	4.9	5.7	
18:0	20.2	19.5	18.9	19.4	21.0	
18:1	36.0	33.5	34.7	33.8	34.9	
18:2	9.2	10.3	11.1	9.8	10.2	
18:3	3.7	4.8	4.2	5.7	4.0	
Subject F						
16:0	34.2	33.8	32.3	33.8	34.1	32.4
16:1	3.4	3.1	3.8	3.1	3.6	3.3
18:0	24.6	23.2	25.0	24.1	22.2	25.2
18:1	28.7	30.2	31.7	28.9	31.2	29.7
18:2	7.4	7.1	6.7	7.9	8.0	6.9
18:3	1.7	1.7	1.0	1.8	1.7	1.6
Subject G						
16:0	27.9	26.4	28.0	26.8	27.0	28.0
16:1	4.0	4.7	5.0	4.6	4.8	3.9
18:0	22.5	24.2	23.1	22.9	24.0	23.3
18:1	33.9	33.9	34.6	34.0	34.3	34.9
18:2	8.1	7.3	7.8	7.6	8.0	9.1
18:3	1.8	2.2	2.0	2.4	1.9	2.1

TABLE 31, STUDY 8 Fatty acid compositions (wt. percentages of the totals) of the plasma triglycerides of subjects given an intravenous infusion of glycerol

	Time after glycerol infusion (min)					
	0	5	10	20	30	40
Subject E						
16:0	24.3	24.1	24.6	24.8	23.3	
16:1	6.7	5.3	6.6	7.1	6.7	
18:0	2.7	2.6	2.7	2.1	3.0	
18:1	49.3	51.0	49.2	48.7	49.1	
18:2	9.7	8.8	10.2	10.1	10.7	
18:3	2.6	2.8	2.7	2.7	2.9	
20:3	0.2	0.2	0.3	0.3	0.2	
20:4	1.0	1.0	1.1	1.1	1.2	
Subject F						
16:0	28.8	28.2	27.8	28.7	29.2	27.9
16:1	4.1	3.8	3.8	3.7	3.9	4.0
18:0	2.5	2.6	2.8	3.6	2.8	3.1
18:1	42.6	41.4	42.6	43.0	43.0	43.9
18:2	14.4	13.3	13.8	13.5	14.4	13.2
18:3	2.0	1.9	2.0	1.9	1.8	1.9
20:3	0.3	0.2	0.3	0.3	0.3	0.3
20:4	1.8	1.6	1.6	1.6	1.6	1.6
Subject G						
16:0	26.1	26.0	28.2	26.0	28.5	28.4
16:1	5.0	6.3	5.5	5.4	6.4	5.9
18:0	3.6	3.1	3.7	3.4	3.5	3.5
18:1	42.6	41.9	43.8	44.0	42.1	41.2
18:2	11.4	11.6	10.9	11.1	10.6	10.4
18:3	3.0	3.5	2.9	2.8	2.4	2.5
20:3	0.4	0.7	0.4	0.3	0.3	0.4
20:4	1.5	1.4	1.6	1.6	1.4	1.4

TABLE 32, STUDY 8 Fatty acid compositions (wt. percentage of the totals) of the plasma cholesteryl esters of subjects given an intravenous infusion of glycerol

	Time after glycerol infusion (min)					
	0	5	10	20	30	40
Subject E						
16:0	9.0	9.4	9.8	9.5	9.5	
16:1	5.0	4.6	5.1	5.0	4.5	
18:0	0.7	0.8	0.8	0.9	1.0	
18:1	24.3	24.0	24.5	23.7	23.8	
18:2	51.0	48.6	49.6	49.6	48.7	
18:3	1.9	1.8	1.4	1.4	1.9	
20:3	0.4	0.5	0.5	0.4	0.6	
20:4	4.2	4.4	4.3	4.4	4.7	
22:6	0.8	0.8	1.0	1.3	1.0	
Subject F						
16:0	11.0	11.7	10.6	10.9	10.9	10.5
16:1	3.6	3.4	3.2	3.3	3.3	3.3
18:0	1.4	1.2	1.1	1.1	1.1	1.0
18:1	22.4	23.7	23.3	23.1	23.4	23.2
18:2	49.1	48.8	49.6	49.0	49.2	49.5
18:3	0.9	0.7	0.8	0.8	0.7	0.8
20:3	0.6	0.6	0.6	0.6	0.6	0.6
20:4	8.0	7.9	7.6	7.8	7.6	7.3
22:6	0.7	0.7	0.9	0.9	1.0	1.1
Subject G						
16:0	11.1	11.3	12.8	11.4	12.3	11.6
16:1	2.8	3.0	3.0	2.6	3.3	3.2
18:0	1.0	1.0	1.1	1.2	1.0	1.0
18:1	21.9	22.2	21.9	22.9	21.7	20.8
18:2	46.5	47.4	47.9	48.6	47.6	47.0
18:3	1.6	1.3	1.6	1.0	1.7	1.6
20:3	0.8	0.6	0.7	0.7	0.7	0.7
20:4	5.9	5.9	5.9	6.2	6.3	6.2
22:6	1.3	1.2	0.9	1.3	1.3	1.4

TABLE 33, STUDY 8 Fatty acid compositions (wt. percentages of the totals) of the plasma phospholipids of subjects given an intravenous infusion of glycerol

	Time after glycerol infusion (min)					
	0	5	10	20	30	40
Subject E						
16:0	30.8	30.0	30.6	28.7	29.1	
16:1	1.1	1.3	1.1	1.2	1.4	
18:0	11.0	11.5	11.3	11.1	11.0	
18:1	15.9	16.1	16.3	16.2	15.9	
18:2	23.5	23.4	23.4	24.6	23.9	
18:3	0.9	0.9	1.0	1.1	1.0	
20:3	2.6	2.7	2.8	2.7	2.8	
20:4	6.9	7.2	7.0	6.9	7.2	
22:5	1.0	1.0	1.0	0.9	1.1	
22:6	4.7	5.4	4.5	4.6	5.1	
Subject F						
16:0	32.9	34.0	32.3	34.2	34.8	33.3
16:1	0.8	0.4	0.5	0.7	0.7	0.7
18:0	11.9	12.4	11.8	11.9	12.0	12.4
18:1	12.2	11.3	12.2	11.8	10.9	11.0
18:2	20.3	19.4	20.5	19.5	19.7	19.6
18:3	0.7	0.7	0.7	0.7	0.6	0.6
20:3	3.2	3.1	3.5	3.4	3.4	3.1
20:4	11.4	11.5	11.5	10.7	10.9	11.0
22:5	0.8	0.9	0.7	0.7	0.9	0.9
22:6	5.0	5.4	5.4	4.9	4.9	4.9
Subject G						
16:0	30.3	28.9	31.3	29.6	31.9	31.6
16:1	0.8	1.0	1.0	1.1	1.0	1.3
18:0	12.7	11.7	12.8	12.7	12.8	12.7
18:1	13.0	12.9	12.8	13.4	13.1	12.9
18:2	17.2	18.2	17.9	18.3	18.3	17.7
18:3	0.8	0.9	0.6	0.8	0.7	0.9
20:3	3.8	3.9	4.1	3.8	3.7	3.8
20:4	9.9	10.9	10.1	10.0	9.8	9.8
22:5	1.2	1.2	1.3	1.4	1.4	1.4
22:6	6.2	6.9	6.6	7.0	6.6	6.6

TABLE 34, STUDY 8 Effect of intravenous glycerol infusion on plasma phospholipid composition (wt. percentage of total phospholipids) (3 subjects; individual values)

	Time after glycerol infusion (min)	
	0	10
Subject E		
Phosphatidic acid	0.0	2.9
Phosphatidyl ethanolamine	2.8	3.0
Phosphatidyl serine	1.3	1.6
Phosphatidyl choline	83.2	76.2
Sphingomyelin	10.1	13.9
Lysophosphatidyl choline	2.6	2.4
Subject F		
Phosphatidic acid	0.0	0.0
Phosphatidyl ethanolamine	3.2	3.2
Phosphatidyl serine	1.8	2.1
Phosphatidyl choline	79.4	75.1
Sphingomyelin	12.9	17.4
Lysophosphatidyl choline	2.7	2.2
Subject G		
Phosphatidic acid	0.0	0.0
Phosphatidyl ethanolamine	2.3	2.6
Phosphatidyl serine	1.8	1.6
Phosphatidyl choline	83.3	80.0
Sphingomyelin	9.8	12.9
Lysolecithin	2.8	2.9

STUDY 9 EFFECT OF INTRAVENOUS GLYCEROL INFUSION ON PLASMA LACTATE AND a-GLYCEROPHOSPHATE CONCENTRATIONS

In view of the reports (Gordon 1957;[39] Fredrickson and Gordon 1958;[35] Trout et al 1960)[124] that high levels of plasma lactate interfere with the Dole (1956)[25] method of determining the concentrations of unesterified fatty acids in plasma, it was decided to investigate the effect of glycerol infusion on plasma lactate concentrations. Since the first step in the metabolism of glycerol involves its phosphorylation with glycerokinase and ATP, it was of interest also to determine the effect of glycerol infusion on the concentration a-glycerophosphate in the plasma.

Eight subjects were each given an intravenous infusion of glycerol as previously described. Blood samples were taken before and at 10 and 40 minutes after the glycerol infusion. In each plasma sample, the concentration of lactate was determined by the method described in the Biochemica Test Combination Cat No 15972 (Boehringer Corporation London Ltd) based on the procedure of Hohorst (1963),[51] and the concentration of a-glycerophosphate was determined by the method of Hohorst (1963).[51]

From the results given in Table 35, it is clear that the glycerol infusion had little or no effect on the concentration of plasma lactate. Therefore, there would seem to be no reason to suppose that increased amounts of lactate passed into the heptane phase during the Dole (1956)[25] extraction of the plasma

samples obtained 10 minutes after the glycerol infusion. On the other hand, the glycerol infusion resulted in a marked increase in the concentration of a-glycerophosphate in the plasma obtained 10 minutes after the infusion, but during the following 30 minutes the concentration of a-glycerophosphate decreased to a level similar to that observed before the infusion. Thus, it may be calculated that during the first 10 minutes after the glycerol infusion, the concentration of a-glycerophosphate in the plasma increased by about 20 u equivalents/litre; in the unlikely event of all of this additional a-glycerophosphate passing into the heptane phase during the Dole (1956)[25] extraction, it would account for only about 5% of the titratable acidity due to the non-fatty acid contaminant.

STUDY 10 THE PROPORTION OF LACTATE TAKEN UP INTO THE HEPTANE PHASE DURING THE DOLE (1956) EXTRACTION OF PLASMA SAMPLES OBTAINED FROM SUBJECTS GIVEN AN INTRAVENOUS INFUSION OF GLYCEROL

Trout et al (1960)[124] found that when plasma was extracted by the method of Dole (1956)[25], about 2.0% of the total plasma lactate passed into the heptane phase. However, the volatility of lactic acid is such that it seemed possible that with the particular procedure used, Trout et al (1960)[124] might have underestimated the concentration of lactic acid inthe unwashed heptane phase. This possibility was investigated in Study 10 in which the partition of lactate between the aqueous and heptane phases of the Dole (1956)[25] extract was determined with (1-^{14}C)-lactate.

Two subjects were each given an intravenous infusion of glycerol as in Study 1 . Blood samples were taken before and 10, 20, 30 and 40 minutes after the glycerol infusion. The concentration of lactate was determined in a portion of each plasma sample by the method of Hohorst (1963)[51]. Carrier-free (1-^{14}C)-lactate (obtained from the Radiochemical Centre, Amersham, Bucks) with a radioactivity of 116,000 dpm was added to a further portion (2 ml) of each plasma sample; these were then extracted by the method of Dole (1956)[25]. Portions of the heptane extracts were counted in 10 ml of organic scintillator (5.0 g of 2,5-diphenyl oxazole and 0.5 g of 1,4 tris-2-(4-methyl-4 phenyl oxazolyl)-benzene per litre of toluene) in a standard glass vial. Radioactivity was determined in a Packard 2425 Scintillation Spectrometer (Packard Instruments Ltd, Illinois, USA). Correction for counting efficiency was made by the use of an external standard (^{226}Ra). Thus, from the specific radioactivity of the lactate in the plasma and the radioactivity in the heptane phase, the percentage of plasma lactate taken up in the heptane phase could be calculated (Table 36).

In agreement with the results of Study 9 , the infusion of glycerol was found to have little or no effect on the concentration of plasma lactic acid (Table 36). In the blood samples taken immediately before the infusion, the values for the percentage of total plasma lactate taken up into the heptane phase (2.2 and 2.9%) are somewhat higher than, but are of the same order as those

(1.8 and 2.2%) obtained by Trout et al (1960) using a very different analytical procedure. It is not clear why the proportion of plasma lactate taken up into the heptane phase varied with time after glycerol infusion; in both subjects maximum values (3.4 and 3.8%) were observed 20 minutes after the infusion. Since the concentration of plasma lactate did not vary, it must be assumed that there was a change in the concentration of some other plasma constituent that exerts some influence on the partition of lactate between the aqueous and heptane phases. It should be noted that the maximum concentrations of glycerol were found in the plasma samples obtained 5 minutes after the infusion.

TABLE 35, STUDY 9 Effect of intravenous glycerol infusion on plasma lactate and a-glycerophosphate concentrations. (8 subjects; mean values with their standard errors)

Plasma concentration	Time after glycerol infusion (min) 0	10	40
lactate (mg/100 ml)	13.7 ±0.38	13.1 ±0.54	12.7 ±0.52
a-glycerophosphate (u moles/100 ml)	4.11 ±1.07	8.40 ±0.94	4.49 ±0.87

TABLE 36, STUDY 10 Proportion of lactate taken up into the heptane phase during the Dole (1956) extraction of plasma samples obtained from subjects given an intravenous infusion of glycerol

(2 subjects; individual values)

	Time after glycerol infusion (min)									
	0		10		20		30		40	
Subject	H	I	H	I	H	I	H	I	H	I
Plasma lactate concn. (mg/100 ml)	10.7	12.9	11.4	13.3	11.0	13.3	10.7	13.6	10.7	12.9
Plasma lactate concn. (mg/2 ml)	0.214	0.258	0.228	0.266	0.220	0.266	0.214	0.272	0.214	0.258
Calculated specific radioactivity of plasma lactate (dpm/ug)	542	449	509	436	527	436	542	427	542	449
Total radioactivity in heptane phase (dpm)	2597	3426	3471	3837	3945	4372	3055	3608	2589	3449
Lactate in heptane phase (ug)	4.7	7.6	6.8	8.8	7.5	10.2	5.6	8.4	4.8	7.7
Percentage of total plasma lactate in heptane phase	2.2	2.9	3.0	3.3	3.4	3.8	2.6	3.1	2.2	3.0

FURTHER INVESTIGATIONS INTO THE IDENTITY OF THE UNKNOWN ACIDIC COMPONENT

Various attempts were made to identify the unknown acidic component that apparently accumulated in the plasma after the intravenous infusion of glycerol, and that passed into the heptane phase during the extraction of the plasma by the method of Dole (1956)[25]. These attempts were not successful and therefore will be described only briefly. Most of the work involved a comparison of the compositons of the heptane phases obtained from the Dole (1956)[25] extraction of the plasma obtained from a number of subjects immediately before or 10 minutes after the intravenous infusion of glycerol. In many instances appropriate heptane extracts were pooled before examination.

SHORT-CHAIN FATTY ACIDS. The possibility that the heptane extracts of the plasma might contain short-chain fatty acids was investigated by gas-liquid chromatography. The method used was essentially that of Cottyn and Boucque (1968)[24]. The chromatography column was of 10% (w/w) Carbowax 20M absorbed onto Chromosorb G, 80-100 mesh (Perkin-Elmer Ltd, Beaconsfield) and was housed in a Pye 104 gas chromatograph fitted with dual flame ionization detectors. The chromatograph was operated with a temperature programmed cycle from 90°C to 130°C, rising by 4°C per minute. This method separated and analysed synthetic mixtures of acetic, propionic, n-butyric, isovaleric and n-valeric acids. Although trace concentrations of these short-chain fatty acids were found in

the heptane extracts of the plasma, the concentrations were too small to affect significantly the titratable acidity of the heptane phase. Moreover, there were no differences in these very small concentrations of short-chain acids in the heptane extracts of the plasma obtained before and 10 minutes after the glycerol infusion. These findings are consistent with those of Study 8 which showed that the intravenous infusion of glycerol did not increase the concentrations of short-chain acids in the unesterified fatty acid fraction of the plasma.

KREBS CYCLE INTERMEDIATES AND RELATED CARBOXYLIC ACIDS. Although Dole (1956)[25] reported that the addition of a-ketoglutaric, β-hydroxybutyric, succinic, citric, malic and pyruvic acids to normal plasma resulted in negligible increases in the titratable acidity of the heptane extracts of the plasma, it seemed necessary to ascertain whether increased amounts of these and related acids passed into the heptane extract of the plasma obtained from subjects given an intravenous infusion of glycerol. Portions of the heptane extracts were analysed by an adaptation of the gas-liquid chromatographic procedures described by Estes and Bachman (1966)[31] and Barnett et al (1968)[11]. Methyl esters of pyruvic, lactic, malonic, fumaric, succinic, malic, a-ketoglutaric, cisaconitic, citric, oxaloacetic and β-hydroxybutyric acids were prepared with methanolic HCl or diazomethane. A mixture of the methyl esters of these acids was well separated by gas-liquid chromatography on a column of 10% (w/w) diethylene glycol adipate on

Chromosorb W. A Pye 104 gas chromatograph was used with dual flame ionization detectors, and the analyses were temperature programmed from an initial column temperature of 88°C to 175°C, rising by 7.5°C per minute. Trace concentrations of pyruvic, lactic, fumaric, succinic, malic, citric, oxaloacetic, a-ketoglutaric and β-hydroxybutyric acids were found in the heptane extracts of the plasma samples obtained before and 10 minutes after the intravenous infusion of glycerol. There was also evidence of a tendency for the concentrations of most of these acids in the heptane phase to increase as a result of the glycerol infusion. In agreement with the results of Study 10, this tendency was most evident with lactic acid. However, calculations showed that the presence of these small amounts of carboxylic acids in the heptane extract of the plasma obtained 10 minutes after the glycerol infusion had a negligible effect on the titratable acidity of the heptane phase.

a-GLYCEROPHOSPHORIC AND RELATED ACIDS. The results of Study 9 showed that the intravenous infusion of glycerol resulted in increased concentrations of a-glycerophosphate in the plasma, but it was calculated that even if all of the increased amount of a-glycerophosphate passed into the heptane phase during the Dole (1956)[25] extraction, it would only account for a very small proportion of the titratable acidity of the heptane. Nevertheless it seemed desirable to confirm that a-glycerophosphate and metabolites derived from it were not contributing to the titratable acidity of the heptane extract

of the plasma obtained 10 minutes after the glycerol infusion. Portions of the heptane extract were analysed by the gas-liquid chromatographic procedure of Hashizume and Sasaki (1966)[47] but neither a-glycerophosphoric, β-glycerophosphoric, 2-phosphoglyceric nor 3-phosphoglyceric acids could be detected in the extracts obtained from the plasma either before or after the glycerol infusion.

BILE ACIDS AND AROMATIC ACIDS. Although there seemed no reason to suppose that the intravenous infusion of glycerol would result in increased plasma levels of aromatic and bile acids, these acids, if present, would be expected to pass into the heptane phase during the Dole (1956)[25] extraction of the plasma.

Portions of the heptane extracts were examined by thin-layer chromatography on Silica Gel G with a solvent system of chloroform-acetic acid (90:10 v/v) according to the method described by Waldi (1965).[130] Bile acids (eg cholic, deoxycholic and dehydrocholic acids) could not be detected when the chromoplates were sprayed with the phosphomolybdic acid, Liebermann-Burchard or vanillin-sulphuric acid reagents (Waldi 1965).[130]

Examination of the heptane extracts by the gas-liquid chromatographic procedures described by Williams and Sweeley (1964)[135] showed the presence of tract concentrations of p-hydroxyphenyl acetic acid and the glycine conjugates of benzoic and phenylacetic acids. However, comparison of the heptane extracts obtained before and 10 minutes after the glycerol infusion revealed no differences in the small concentrations of these aromatic acids. In any event, it was

calculated that these very small amounts of aromatic acids would have a negligible effect on the titratable acidity of the heptane phase.

PLASTICIZER. Portions of the heptane phase derived from the Dole (1956)[25] extraction of the plasma obtained immediately before or 10 minutes after the glycerol infusion were examined by thin layer chromatography on Kieselgel G plates with a solvent system of hexane-diethyl ether-formic acid (80:20:2, by vol). In addition to unesterified fatty acids, the heptane extracts were found to contain cholesteryl esters, free cholesterol, triglycerides and phospholipids, and as might be expected from the results of previous experiments, the infusion of glycerol decreased the proportion of unesterified fatty acids in the heptane phase, but did not alter the proportions of the other plasma lipids. However, examination of the chromatograms of the heptane extracts of the plasma obtained 10 minutes after the glycerol infusion revealed the presence of a component that was not present in the extracts of the plasma obtained before the glycerol infusion. With the solvent used, this additional component migrated to a point on the chromatoplates midway between the unesterified fatty acid and cholesterol bands. Some difficulty was encountered in attempting to identify this component until it was discovered that the component was derived from the 50 ml polypropylene syringes used inthe intravenous glycerol infusions. When a 10% solution of glycerol was passed through such a syringe

and portions of the resulting glycerol solution extracted by the Dole (1956)[25] procedure, it was discovered that the heptane phase contained a compound, the chromatographic properties of which were identical to those of the additional component that was present in the plasma obtained 10 minutes after the intravenous infusion of glycerol. This finding suggested that the compound was a plasticizer used in the manufacture of the plastic syringe, and that it had passed into the blood stream with the infused glycerol solution. No attempt was made to identify this plasticizer completely since it was found to possess no titratable acidity and therefore could not account for the differences in the plasma concentrations of unesterified fatty acids obtained by the different analytical procedures. However, on thin layer plates of Kieselgel G and with solvent systems of iso-octane-ethyl acetate (90:10 v/v) or hexane-diethyl ether-formic acid (80:20:2, by vol) the chromatographic properties of this plasticizer were found to be similar to those of an authentic sample of di-n-butyl phthalate. In the manufacture of plastics, various esters of O-phthalic acid are used as plasticizers and these may pass into aqueous solutions that come into contact with such plastic materials. For example, di octyl phthalate has been shown to occur in samples of commercial milk[22] taken from plastic containers (Cerbulis and Ard 1967). Marcel and Noel (1970)[87] have identified dihexyl-2-ethyl phthalate in human blood that had been stored for a few days in a plastic transfusion pack; the concentration of the phthalate ester increased as the time of storage in the plastic pack increased.

DISCUSSION

As might have been predicted from the known gluconeogenic properties of glycerol (Cahill 1966[16]; Senior and Loridan 1968[112]; Exton 1972[33]), the results showed that the intravenous infusion of glycerol resulted in a decrease in the plasma unesterified fatty acid levels when these were determined by the specific methods of Christie et al (1970)[23] and Duncombe (1964)[28]; this finding was supported by the qualitative examination of the plasma lipids by thin-layer chromatography. In contrast, the intravenous infusion of glycerol was found to increase the concentrations of plasma unesterified fatty acids when these were determined by the less specific method of Dole (1956)[25]. This discrepancy between the values obtained by the method of Dole (1956)[25] on one hand, and those obtained by the methods of Duncombe (1964)[28] and Christie et al (1970)[23] on the other, was greatest in the plasma samples obtained 10 minutes after the glycerol infusion. In the plasma samples obtained 10 minutes after the glycerol infusion in Study 6, the true concentration of unesterified fatty acids was about 360 u equiv/l, whereas that determined by the method of Dole (1956)[25] was about 1000 u equiv/l; this discrepancy of 640 u equiv/l amounted to a positive error of 180% in the Dole (1956)[25] procedure. The addition of glycerol to plasma in vitro, up to concentrations of 10 mg/100 ml was found to introduce only a small positive error of about 2% in the values for the concentration of unesterified fatty acids obtained by the Dole (1956)[25] method.

Since the glycerol used in the infusion experiments was found to possess no titratable acidity, it seems reasonable to assume therefore that the intravenous infusion of glycerol resulted in the accumulation in the plasma of an unknown acidic component that passed into the heptane phase during the Dole (1956)[25] extraction procedure, and was thus titrated as "unesterified fatty acids". The fact that, in the plasma samples obtained before the glycerol was infused, there was good agreement between the values for the concentrations of unesterified fatty acids determined by all three analytical procedures indicated that this unknown acidic component was absent from, or was presnet in only trace amounts in normal plasma. It was of interest to note that the unknown acidic component was only partially removed from the heptane extract by treatment with 0.05% H_2SO_4 (Table 27) as recommended inthe modification of the Dole (1956)[25] method proposed by Trout et al (1960).[124] It is also of interest to record that these findings are not confined to experiments with human subjects; a pattern of results similar to that given in Table 26 was obtained from an analogous investigation (unpublished) in which sheep were given intravenous infusions of glycerol.

The results would be consistent with the unknown acidic component being a product of glycerol metabolism. The glycerol infused into the blood stream could be (a) oxidatively catabolized for energy production, (b) utilized for glycerolipid synthesis or (c) utilized for gluconeogenesis. The initial step

in all three of these processes in the reaction of the glycerol with ATP and glycerolinase to form a-glycerophosphate. The intravenous infusion of glycerol did, in fact, give rise to a small increase in the plasma concentration of a-glycerophosphate (Study 9), but none of this a-glycerophosphate appeared to pass into the heptane phase when the plasma was extracted by the procedure of Dole (1956)[25]. The oxidative metabolism of glycerol via the glycolytic pathway involves the conversion of a-glycerophosphate to dihyroxyacetone phosphate, glyceraldehyde phosphate, 1,3-diphosphoglycerate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenol pyruvate and then pyruvate. These acidic intermediates are highly polar compounds and it would seem unlikely that more than trace amounts of them would pass from the aqueous phase to the heptane phase during the Dole (1956)[25] extraction of the plasma. Gas-liquid chromatographic analyses confirmed that the heptane extracts contained neither 2-phosphoglycerate nor 3-phosphoglycerate, and only trace concentrations of pyruvate. Further oxidative metabolism of pyruvic acid via the Krebs cycle would involve the formation of citrate, cis-aconitate, a-ketoglutarate, succinate, fumarate, malate acid and oxaloacetate, but as might have been predicted, none of these hydrophilic intermediates could be detected in more than trace concentrations in the heptane extracts of the plasma samples. If a-glycerophosphate was utilized for glycerolipid synthesis, it was conceivable that acidic phospholipids might accumulate in the

plasma and pass into the heptane phase during the Dole (1956)[25] extraction. Phosphatidic acid seemed to be the only acidic phospholipid that could have any appreciable effect on the titratable acidity of the heptane extracts. Phosphatidic acid is not normally present in plasma in more than trace concentrations, but it appeared feasible that these concentrations might increase as a result of the glycerol infusion. However, in only one of the subjects examined wa s the intravenous infusion of glycerol found to increase the concentration of a-glycerophosphate in the plasma. In this subject, the increased levels of plasma phosphatidic acid were too small to have any appreciable effect on the titratable acidity of heptane extract of the plasma. For reasons that are at present obscure, the intravenous infusion of glycerol resulted in a change in the phosphatidyl choline:sphingomyelin ratio in the plasma. This change in ratio was also reflected in the composition of the phospholipids extracted from the plasma by heptane. Nevertheless, when the plasma lipids extracted into the heptane phase were chromatographed on columns of silicic acid, it was found that the titratable acidity due to the phospholipid fraction alone was not altered by the glycerol infusion. It seems inconceivable that increased plasma concentrations of any of the highly hydrophilic intermediates inthe metabolic conversion of a-glycerophosphate to glucose, namely, dihydroxyacetone phosphate, glyceraldehyde-3-phosphate, fructose 1,6-diphosphate, fructose-6-phosphate and glucose-6-phosphate could introduce large positive errors in the determination of plasma unesterified fatty acid levels by the Dole (1956)[25] procedure.

According to Trout et al (1960)[124] plasma lactate interferes with the determination of the concentration of plasma unesterified fatty acids by the method of Dole (1956)[25]. In view of this, the concentrations of lactate were determined in the plasma of the experimental subjects, in spite of the fact that there was no reason to suppose that the intravenous infusion of glycerol would increase plasma lactate levels (Williamson, Veloso, Ellington and Krebs 1969)[136]. Studies 9 and 10 confirmed that the glycerol infusion was without effect on plasma lactate levels. In the plasma samples obtained immediately before the glycerol infusion, about 2.5% of the total plasma lactate was taken up into the heptane phase during the Dole (1956)[25] extraction procedure; this value was very similar to that reported by Trout et al (1960)[124]. However, although the total plasma lactate levels remained constant, the percentage of plasma lactate taken up into the heptane phase appeared to increase with time after the glycerol infusion; values of 3.2% and 3.6% were observed at 10 and 20 minutes respectively (Table 36). No explanation can be put forward for this increased transfer of lactate from the aqueous phase to the heptane phase. However, further experiments (unpublished) in which glycerol was added to sheep plasma <u>in vitro</u> have shown that the percentage of plasma lactate passing into the heptane phase during the Dole (1956)[25] extraction of the plasma increased as the concentration of glycerol in the plasma increased. These findings with sheep plasma <u>in vitro</u> are not entirely consistent with those obtained with human plasma <u>in vivo</u>, in which the concentration of glycerol was greatest at 10

minutes, but the uptake of lactate into the heptane phase was greatest at 20 minutes. In any event, the relatively small amounts of lactate taken up into the heptane phase cannot account for discrepancies between the results for the plasma unesterified fatty acid levels obtained by the various analytical techniques. For example, the concentration of plasma lactate 10 minutes after the glycerol infusion was 13.1 mg/100 ml (Table 35) which amounts to 14.54 u equiv/l; 3.2% of this (Table 36), which amounts to 46.52 u equiv/l, passed into the heptane phase during the Dole (1956)[25] extraction procedure. In the plasma samples obtained 10 minutes after the glycerol infusion, the difference between the true concentration of unesterified fatty acids and that determined by the Dole (1956)[25] method amounted to 640 u equiv/l. Thus, the lactate passing into the heptane phase can only account for 7.3% of this discrepancy.

The heptane phase was found to contain trace amounts of various other acidic omponents such as short chain acids, aromatic acids and Krebs cycle intermediates etc. Calculations show that the trace concentrations of all of these acidic components plus the concentration of lactate in the heptane extracts of the plasma samples obtained 10 minutes after the glycerol infusion accounted at the most for only about 14% of this discrepancy of 640 u equiv/l. The greater part of this discrepancy must therefore be due to an unidentified acidic compound that accumulated in the plasma as a result of the glycerol infusion. At present it is difficult to speculate on the nature of this compound but a high volatility

or a high lability is suggested by the failure of the various chromatographic and other techniques that were used in the attempts to isolate and characterise the compound (Noble, Lorimer, Moore and Lawrie 1975).[93]

The discovery in the plasma obtained 10 minutes after the glycerol infusion of a plasticiser derived from the plastic infusion syringe calls for a thorough investigation of the solubilities of such plasticisers in the various aqueous solutions that are administered to patients by injection or infusion; contamination could occur during storage in plastic containers or during administration with plastic equipment. It is also important that more information should be obtained about the metabolism, excretion and possible toxicity of such plasticisers.

CHAPTER FIFTEEN

DISCUSSION

Several opinions have been expressed as to why cigarette smoking and CHD may be linked.

The older view (von Ahn 1960)[1] was that prolonged cigarette smoking produced recurrent episodes of coronary arterial constriction resulting eventually in persistent coronary artery disease. A limited amount of evidence does show that cigarette smoking produces either a reduction or at least no change in coronary flow in those with pre-existing CHD compared to a possible increase in those with normal vessels. Failure to increase myocardial blood flow consequent upon increased demands for oxygen may result in relative ischaemia and cause dysrhythmias. These blood flow studies were, however, carried out using external praecordial counting after the intravenous injection of a radio-active isotope and have been difficult to confirm or refute. Overall the method is not regarded as satisfactory. Furthermore, coronary angiography was not available to demonstrate significant differences between the groups in terms of coronary artery disease.

Emphasis has also been placed on the role of cigarette smoking as an aetiological factor in the development of coronary atheroma and the work of Strong and Eggen (1970)[120] and Auerbach, Hammond, Garfinkel and Kirman (1971)[6] would tend to support the view that prolonged exposure to cigarette smoke increases the thickness of coronary arteries, causes excess atheroma and reduces the lumen of the vessels.

There is also widespread epidemiological evidence linking cigarette smoking and CHD (Royal College of Physicians 1971,[116] U.S. Department of Health, Education and Welfare 1972).[121] It is said, for example, that around 52,000 deaths occur each year in Britain as the result of cigarette smoking (Department of Health and Social Security 1972) and that of these deaths approximately 50% are due to cardiovascular disease, mainly CHD. Several studies have also suggested that not only do cigarette smokers have an increased incidence of myocardial infarction, but are also much more at risk from sudden death. Kannel, Castelli and McNamara (1968)[56] reported that myocardial infarction occurred twice, but sudden death five times more frequently in heavy smokers than in non-smokers. In fact, sudden death seems to be rare in non-smokers (Turner and Ball 1973).[125] Spain, Siegel and Bradess (1973)[118] commented that this increased risk of sudden death now seems to be affecting women as well as men and may be due to the fact that heavy smoking (more than 20 cigarettes daily) has become more common in women. Their conclusion was that 12 years ago 12 men died suddenly compared to a single sudden death in women. The ratio has decreased and now approximates 4 men and 1 woman. Sixty-two per cent of women dying suddenly are heavy smokers, and the excess mortality cannot be related to heart size or hypertension. This suggests a possible acute metabolic effect of cigarette smoking harmful to the myocardium in subjects perhaps

already predisposed because of the presence of considerable, though unsuspected, CHD. It seems likely that the myocardium of certain individuals with CHD is more vulnerable than others in light of the poor relationship between occlusive arterial disease and myocardial fibrotic scarring (Morris and Crawford 1958)[88] and also because sudden death is distributed similarly among those with varying degrees of coronary atherosclerosis. (Kuller, Cooper and Perper 1972).[70] The problem of the "vulnerable myocardium" has been discussed by Anderson (1973)[2] and Oliver (1973)[94] and will be referred to later.

Kershbaum and his associates have, in a series of papers (1962,[59] 1967,[62] 1968)[58] shown that cigarette smoking produces a rise in plasma FFA and that this rise appears higher in subjects who have sustained a myocardial infarction (and who are thus likely to have widespread coronary artery disease), than in those with no clinical evidence of CHD. These studies were, however, in some respects incomplete. Subjects were investigated at varying times after myocardial infarction, and from their data it is not possible to be sure whether or not the apparently enhanced response is present for only a short time after infarction or whether a persistent long-term effect occurs. Nor was it shown whether the enhanced release of FFA took place in all subjects with coronary heart disease or only in those who had sustained a myocardial infarction. The possible clinical significance of this rise in FFA took on new importance with the reports by Oliver, Kurien and Greenwood (1968)[95]

and Kurien, Yates and Oliver (1969)[73] that FFA levels increased after myocardial infarction, that highest levels of FFA were associated clinically with an increased tendency to dysrhythmias and that experimental elevation of FFA levels could cause dysrhythmias. The FFA-dysrhythmia relationship is not without controversy (Opie, Thomas, Owen, Norris, Holland and Van Noorden 1971:[96] and Gupta, Jewett, Young, Hartog and Opie (1969).[42] Lewis (1974)[81] measured urinary catecholamines in a small series of smokers and non-smokers who had sustained a recent infarction. Urinary catecholamines and dysrhythmic episodes were raised and more frequent respectively in those who were smokers. It was felt unlikely that the raised catechols were a consequence of the dysrhythmia since in most patients the finding of raised urinary catecholamines preceded the development of the dysrhythmia. In addition this effect was not likely to be due to an acute effect of nicotine inhalation since smoking had been discontinued some time before collections were made. It was suggested that there was possibly a residual condition of enhanced adrenergic activity due to chronic stimulation by nicotine. Rowe, Neilson and Oliver (1975)[108] have now demonstrated that the use of a nicotinic acid analogue after infarction can lower the incidence of dysrhythmias and that this reduction is associated with a lowering of FFA levels to near normal values.

Because of these possible links between CHD, cigarette smoking, FFA release and sudden death the series of investigations

in this study were undertaken to explore and clarify the effect of cigarette smoking in various groups of subjects and to see whether or not there was any difference in the type and fate of FFA produced as a consequence of cigarette smoking.

Care was taken to ensure that the "model" used for assessment of cigarette smoking was satisfactory. The stimulus of insertion of a needle either intravenously or subcutaneously may in itself cause a transitory rise in FFA levels. In addition FFA levels may vary according to diet, time of day and previous exercise. Table 3 shows that the method used was satisfactory as a means of obtaining stable values for FFA and ketone body levels. An intravenous cannula, previously inserted under local anaesthesia, was used for periodic sampling obviating the need for repeated venepuncture; subjects fasted overnight and were studied in the semi-prone position without any prior exertion. Under these conditions, serial FFA and ketone body levels suggest a satisfactory steady state. Following subcutaneous injection of 0.5 ml of normal saline there was a small but definite rise of approximately 10% in FFA values and 20% in ketone body values. These changes were similar in both control subjects and in those studied 3 weeks following myocardial infarction, suggesting that a noxious stimulus produced a positive but equal response in these 2 groups of subjects. It was also considered possible that the act of smoking rather than the pharmacological consequences

of inhaling cigarette smoke might contribute to the metabolic response perhaps by psychological stimulation of the sympathetic adrenomedullary system. Accordingly, low nicotine cigarettes were smoked by both control and post-infarction subjects. There was no significant increase in the levels of any of the variables studied which suggested that the act of cigarette smoking by itself was not responsible for any pronounced metabolic effect. This was specially important with regard to those subjects studied following recent myocardial infarction. All had smoked more than 20 cigarettes daily before their infarction but subsequently had been advised and encouraged to stop smoking. They had not done so but were, of course, aware of disapproval for their action in restarting cigarette smoking, albeit at a much lower level. Nonetheless, their response to smoking low nicotine cigarettes was similar to controls. It should, however, be noted that in all series of experiments the recent myocardial infarction group have a higher resting level of FFA. This would be in keeping with enhanced sympathetic adrenomedullary activity following infarction - perhaps as a consequence of "stress".

The studies reported in Tables 6A and B suggested that those subjects with a recent myocardial infarction had a significantly greater response than control subjects in terms of incremental and total FFA release after smoking. This was associated with increased production of ketone bodies but no definite changes were seen in glucose or insulin levels. It was

decided to further investigate this response in two groups of subjects; with a history of angina pectoris and who had ischaemic changes on their ECG but who had no evidence of previous infarction and a group presenting with clinical and angiographically demonstrable peripheral vascular disease (PVD). Such subjects are of course known to have a high prevalence of CHD but those studied gave no history of angina and had a normal ECG suggesting that their clamant problem was atherosclerosis of the peripheral vessels. These groups were studied in a similar way to the post-infarction subjects and the results shown in Table 7 and Figure 3. Those with angina had rises in FFA and ketone bodies comparable to those found in control subjects but less than those that occurred when post-infarction subjects smoked. Subjects with PVD had a rise in FFA levels after smoking, but these increases were smaller at each time interval than in control subjects or in those with angina. A possible reason for this difference is that persons with PVD are known to be remarkably heavy smokers and those studied were no exception. They had on average daily consumption of 30-40 cigarettes compared with approximately 20 daily for the control and angina group. Habituation to cigarette smoking could be partly responsible for the reduced response, although it should be noted that basal values of FFA were comparable. When daily cigarette consumption is considered then the total daily increase in FFA

as a consequence of smoking is similar for each group.

The overall impression is thus that subjects with a recent myocardial infarction have a greater than normal increase in FFA levels and it has been suggested (Kershbaum 1968)[58] that such a response is the consequence of myocardial infarction, and is likely to be persistent. Because of the unsatisfactory nature of the evidence on which the statement of prolonged effect is based, it was decided to investigate this further in 6 male subjects one year after infarction. All had continued to smoke with an average daily consumption of 15-20 cigarettes. They were admitted to hospital 2 days beforehand, fasted and kept in bed on the morning of the investigation which was conducted as before. Basal levels of FFA were lower at a mean of 696 $\pm$ 199 ueq/l compared to 869 $\pm$ 187 ueq/l in the recent infarction group. Smoking 2 normal cigarettes again resulted in a rise in the levels of FFA and ketone bodies but this increase was similar to that found in control subjects and those with angina, and was thus less than those studies shortly after infarction. The enhanced response to cigarette smoking would seem to be a temporary rather than a persistent phenomenon. It would appear to be related to recent infarction rather than to the presence of CHD either manifest as angina or in patients who have recovered from a previous infarction.

This finding may have relevance to the clinical course of CHD. Cigarette smoking is known to be associated with an

increased risk of myocardial infarction and especially with sudden death (Doyle 1970;[26] Friedman, Manwaring, Rosenman, Donlon, Oretego and Grube 1973).[37]

In a series of male patients presenting with primary myocardial infarction Wilhelmsson, Elmfeldt, Veden, Tibblin and Wilhelmsen (1975)[134] found a high prevalence of cigarette smokers prior to infarction. Those who subsequently stopped smoking had only half the rate of non-fatal recurrences and half the cardiovascular mortality rate of those who continued to smoke. In addition, Gordon, Kannel, McGee and Dawber (1974)[41] reported from Framingham that cigarette smoking increased the attack rate from CHD while those who stopped smoking after entry to the study had only half the CHD attack rate of those continuing to smoke. In addition, elevation of FFA levels are known to be associated with tachydysrhythmias both in clinical and experimental studies. The elevations in plasma FFA levels inducing dysrhythmias in dogs are higher than the levels of plasma FFA occurring in post myocardial infarction (Oliver, Kurien and Greenwood 1968;[95] Kurien, Yates and Oliver 1971)[74] unless in situations where heparin has been administered (Rutstein, Castelli and Nickerson 1969).[110] It could be suggested that these experimental studies are not necessarily relevant to the clinical situation. Other evidence, however, is in keeping with the hypothesis that increase in FFA flux is detrimental to those with a compromised circulation. The patient recovering from a myocardial infarction is likely to have had previous episodes

of symptomatic or even asymptomatic myocardial ischaemia and could have high myocardial concentrations of triglyceride (Wartman, Jennings, Yokoyama and Clabaugh 1956).[131] Catecholamine stimulation of the myocardium - brought about by nicotine from cigarette smoking - could lead to lipolysis of stores of extracellular myocardial triglyceride (Kruger, Leighty and Weissler 1967)[69] with consequent high concentration of intracellular FFA even in the presence of relatively small plasma FFA increments. In addition, Leon and Abrams (1971)[79] have pointed out that natural catecholamines and sympathomimetic drugs may cause cardiac dysrhythmias by increasing automaticity, by stimulating ectopic pacemaker activity and perhaps also by electrophysiological effects leading to re-entrant activity. Furthermore, cardiac work and oxygen requirements are increased.

It seemed logical, therefore, to investigate the possibility of modifying the FFA response to cigarette smoking by beta-adrenoreceptor blockade. Pilkington, Lowe, Robinson and Titterington (1962),[101] studied the early beta-adrenoreceptor blocking drug, nethalide, and showed that this prevented, in animals, the rise in FFA produced by adrenaline. The drug chosen for the present study was propranolol, a non-selective beta-adrenoreceptor blocking agent with reported blocking action on the B1 receptors responsible for lipolysis. A comparison was made of the effect of intravenous placebo (saline) and propranolol in a dose of 0.05 mg/kg bodyweight on FFA and ketone body levels after smoking 2 normal cigarettes (Table 13 and Figure 10). The rise in FFA

levels produced by cigarette smoking was abolished by propranolol. It is interesting to note that the abolition of the FFA response was associated with a considerable reduction but not disappearance of the ketone body response. It may be that an additional metabolic pathway is involved.

Short term administration of beta-adrenoreceptor blocking agents is, therefore, associated with reduced FFA response to cigarette smoking. Long-term studies of the effect of beta-adrenoreceptor blockade on mortality and incidence of sudden death in post-infarction patients and in cigarette smokers are therefore awaited with interest. If they prove to be beneficial then one mode of action would be preventing harmful effects of FFA on an already compromised myocardium and such therapy would appear to be logical.

A comment should also be made on these overall results compared to those reported by Kingsbury and Jarrett (1967).[65] Contrary to their findings it was not possible to show any change in blood glucose levels after smoking 2 normal cigarettes. These authors had suggested also that the smoking of 2 cigarettes would, in certain subjects, produce a rise in plasma insulin. In none of the studies reported here, however, was there any evidence to suggest that 2 cigarettes were a sufficient stimulus to cause any increase in insulin levels. Although ketone bodies are known to increase insulin values (Madison, Mebane, Ungar and Lochner 1964)[86] this is usually associated with a fall in FFA levels and is likely to represent a different mechanism. The results of Porte, Graber, Kuzuya and Williams (1968)[102]

also suggest that adrenaline administration may inhibit insulin response even in the presence of hyperglycaemia. Cigarette smoking is associated with nicotine induced sympathetic and adreno-medullary stimulation. In the short term at least the effect of cigarette smoking does not seem to be the consequence of repeated stimuli to insulin secretion causing alteration in carbohydrate metabolism.

The main metabolic processes involved for mobilised FFA are

(a) oxidation to carbon dioxide or ketone bodies

(b) incorporation into VLDL triglyceride

(c) storage as liver triglyceride

From the data presented, the rise in FFA found after smoking was associated with increased ketone body production (Figure 5) with no evidence of short term increase in triglyceride levels (or of cholesterol). No comment can be made with regard to hepatic accumulation of triglyceride. These findings occurred in all groups of subjects studied.

Considerable interest has been expressed in recent years in the classification of hyperlipoproteinaemias developed by Fredrickson, Levy and Lees (1967).[36] Using age related values for total cholesterol, triglyceride and, if necessary, cholesterol content of low density lipoprotein, subjects can be divided into a variety of "types". The commonest abnormalities are Type II and Type IV hyperlipoproteinaemia. Type II has a raised

total cholesterol or low density lipoprotein cholesterol level whereas Type IV has a raised triglyceride level. It was felt that the level of response to cigarette smoking might be associated with the underlying lipoprotein pattern. Subjects were therefore divided into those with normal lipids, those with probable Type II and those with probable Type IV abnormality. Fasting levels were used but ultracentrifugation was not available to permit measurements of low density lipoprotein cholesterol. In general terms, there was no major difference in the levels of FFA or ketone bodies that developed after smoking in each of the groups studied. The FFA levels reached in Type IV were higher than those in normal subjects or those with Type II but were not significantly different when expressed as a percentage increase and compared to fasting levels since fasting FFA in the Type IV group were higher than in the others. Overall these results indicate the differences in FFA and ketone body responses to smoking are not a function of the underlying cholesterol level. The total (but not percentage) increase in FFA after smoking has a relationship to basal FFA level. These findings are similar to those reported in a different context by Nestel (1964)[92] when levels of FFA reached after noradrenaline infusion were found to be related to triglyceride levels.

It was considered that smoking 2 cigarettes might be an insufficient stimulus to promote changes in glucose and insulin as distinct from raising FFA and ketone levels. Accordingly, subjects were asked to smoke 6 low nicotine or 6 normal nicotine content cigarettes over a 3 hour period with measurement of

the indicated variables before and after smoking. It was not considered feasible to ask subjects who had sustained a recent myocardial infarction to smoke to this extent and accordingly the study compared control, angina and PVD groups of subjects. The smoking of 6 low nicotine cigarettes failed to produce any significant change in levels of FFA, ketone bodies or glucose. When 6 normal nicotine content cigarettes were smoked and when the mean values for each group were compared there was a significant increase in levels of glucose, FFA and ketone bodies. The increases in the control, PVD and angina group were comparable. There was no tendency for any one group to have an excessive response. More interesting results emerged when the subjects were studied separately rather than in groups (Table 15). Two main patterns were seen. Nine subjects had a marked rise in blood glucose while in 7 the glucose level either fell or remained relatively unchanged. The FFA and ketone body response was similar in each group and did not appear to be related to changes in glucose. It has been suggested that ketone bodies may represent a stimulus to insulin production. However, in only two of those showing a fall was there any major change in insulin levels. In one subject with PVD the insulin levels rose from 24 to 81 units with an increase in glucose sugar from 74 to 120 mg/100 ml while in the other the insulin changes were 26 to 40 with glucose rising from 89 to 107 mg/100 ml. These results are in contra-
distinction 65
~~indication~~ to those reported by Kingsbury and Jarrett (1967). They found that cigarette smoking (unlike adrenaline administration)

tended to produce either no change or a fall in blood glucose. They also found 5 subjects in whom the immunoreactive insulin level increased although this could not be related to changes in glucose levels. These authors suggested that more than one response to cigarette smoking might occur such as insulin hypersecretion due to vagal stimulation. In their study, adrenaline produced a rise in blood glucose as well as a rise in insulin levels. In the present study, however, levels of FFA were not different in those who increased insulin levels ~~as~~ when compared to those who did not, whereas excess catecholamine release would have been thought to be associated with a demonstrable difference in FFA levels.

Kershbaum, Bellet, Jiminez and Feinberg (1966)[60] reported that urinary catecholamine values increased after heavy cigarette smoking and it was decided to compare urinary free adrenaline and noradrenaline in the various groups of subjects studied. The fasting levels of FFA were significantly higher in the post-infarction group and it was felt this could be a residual consequence of the "stress" of infarction. It might be that the increased amounts of FFA released after smoking were due either to increased catecholamine release or to enhanced response to nicotine induced sympathetic-adrenomedulla stimulation. However, urinary catecholamine values represent only a small proportion of released catecholamine (probably about 3%) and the smoking of 2 cigarettes did not provide sufficient stimulus to allow differentiation between the

groups in terms of catecholamine release. No significant changes were found in levels of urinary adrenaline and noradrenaline. A study of urinary catecholamine levels was also made using a 3 hour period of urinary collection and the more severe stimulus of smoking 6 normal nicotine content cigarettes. There was no change in urinary adrenaline or noradrenaline after smoking 6 low nicotine content cigarettes, but after normal cigarettes there was a significant rise in total urinary catecholamines due to increase in both adrenaline and noradrenaline probably reflecting stimulation of both the sympathetic nervous system (noradrenaline) and adrenal medulla (adrenaline). There was, however, no difference between control, angina or PVD groups. This is in keeping with the similar increments in FFA found in these groups. The possible relationship between catecholamine release and adverse circulatory and metabolic effects has attracted considerable attention. Carruthers (1969)[21] commented on the relationship between aggression and atheroma. Adrenaline and noradrenaline both cause a rise in FFA by lipolysis of triglyceride in adipose tissue. In general noradrenaline evokes a greater response than adrenaline and obese subjects - having higher fasting levels of FFA - tend to have greater absolute increases in FFA levels; similar findings to those found in the current study. Carruthers suggested that cigarette smoking and reactions to stress such as driving provoke similar responses. He believes that in the absence of intermediate metabolic requirements, FFA are converted in the liver

to triglyceride and that this newly formed triglyceride may be deposited in the arterial wall. However, Oliver, Kurien and Greenwood (1968),[95] could find no correlation between FFA and triglyceride levels although they did not follow serial triglyceride values. Hill and Wynder (1974)[48] studied the effect of smoking 2 cigarettes of increasing nicotine content on levels of FFA, cortisol and catecholamines in blood samples taken 1 and 20 minutes after smoking. They found an increase in FFA levels and a rise in adrenaline though not in noradrenaline. In addition, the smoking of high nicotine content cigarettes was accompanied by a rise in cortisol values. Such results have not always been confirmed. Klensch (1967)[66] reported an increase in noradrenaline as well as adrenaline and our own results would suggest that smoking 6 cigarettes is sufficient stimulus to produce demonstrable rise in urinary adrenaline and noradrenaline. Nor has a change in cortisol values been consistently found. Ballantyne and Lorimer (1975 in preparation)[8] found a rise in FFA but no changes in plasma cortisol levels, in a series of samples taken between 15 and 60 minutes after smoking. It may be that the different time intervals of sampling after smoking are contributory to the difference in results. Certainly, in our study, nicotine stimulation of catecholamine induced lipolysis was not associated with cortisol changes and it seems unlikely that cortisol could have been sensitising the myocardium to the effects of catecholamines (Oliver, Kurien and Greenwood 1963).[95] Hill and Wynder[48] were also unable to relate increase in adrenaline values with increased FFA levels. More indirectly, this has not been our experience. Increased numbers of cigarettes

smoked produced greater increases in FFA, presumably as a consequence of greater stimulation of lipolysis.

The effect of small doses of subcutaneous adrenaline on levels of glucose, FFA, ketone bodies and insulin were studied in control subjects and in subjects 3 weeks after infarction. The adrenaline given was insufficient to alter blood glucose, but was sufficient to cause significant increases in FFA levels. These increases were greater in the post-infarct group than in control subjects. Thus both the smoking of similar numbers of cigarettes and injection of equivalent amounts of adrenaline produced an enhanced FFA response in those studied shortly after infarction. It is suggested that a form of catecholamine sensitivity could have been induced or present in these subjects possibly as a consequence of stress associated with their infarction.

Measurements of levels of FFA before and after smoking reflect changes in total amounts of FFA but does not indicate any possible relative changes in the proportion of individual fatty acids. It was decided to use the combination of thin layer chromatography (TLC) and gas-liquid chromatography (GLC) to measure levels of individual fatty acids both in the free or un-esterified moiety and as contained in triglycerides. This was done before and after smoking. The results are shown in Tables 17A and B. The fatty acids of highest concentration both in the FFA and in triglyceride were palmitic (<16) and oleic. Following smoking, there was no change in the proportions found suggesting

that all FFA rose uniformly and that there was no individual fatty acid that could be identified as having a more marked rise of possible clinical significance. Nor did any change occur in the proportions present in the triglyceride fatty acids to suggest preferential accumulation of any individual fatty acid. Tracer studies would, however, be needed for complete evaluation.

It seems likely, therefore, that the rise in FFA as a consequence of cigarette smoking is generalised rather than particular.

Studies of lipolysis have shown that lipids are mobilised from adipose tissue in the form of FFA and that this release of FFA requires lipolysis of the stored triglycerides. In vitro studies have demonstrated that adipose tissue can release glycerol to the incubation medium indicating complete lipolysis of glyceride to FFA and free glycerol (Leboeuf, Flinn and Cahill 1959).[77] In vitro studies suggest that this glycerol is not re-utilised to any great extent in adipose tissue as a precursor of glyceride-glycerol in the esterification of fatty acids to glycerides (Shapiro, Chowers and Rose 1957).[113] In this respect glycerol apparently differs from the liberated fatty acids which are readily taken up and re-esterified within the tissue. The considerable interest in FFA has not been accompanied by a similar interest in glycerol levels. It has, however, been shown that the addition of adrenaline to adipose tissue incubated in vitro increases the release of FFA and glycerol to

the medium (Leboeuf, Flinn and Cahill 1959).[77] Carlson and Oro (1963)[19] studied the relationship between concentrations of FFA and glycerol and found, in a group of human volunteers, a correlation between fasting levels of FFA and glycerol and that noradrenaline administration increased both FFA and glycerol. Their studies also suggested that the main effect of noradrenaline was on lipolysis and not on the re-esterification process.

Studies on glycerol turnover (Tibbling, Cederquist and Lundquist 1969;[123] Senior and Loridan 1969)[112] all indicate that a single exponential can express the rate of disposal of intravenously administered glycerol. Possible mechanisms for the metabolism of glycerol include

(a) gluconeogenesis

(b) oxidative catabolism for energy production

(c) glycero-lipid synthesis

It is believed that glycerol is phosphorylated in the liver and that around 70% of the triosephosphate is converted to glucose and glycogen.

The effects of cigarette smoking on lipolysis in various groups of subjects have been mainly confined to FFA measurements and have not discussed the concomitant glycerol release. Little is known as to whether or not glycerol removal from the plasma differs in various clinicopathological conditions or indeed whether such removal can be influenced by smoking.

Studies of glycerol removal in various groups of subjects indicated that graphic display of the data on semi-logarithmic paper permitted a single exponential to be drawn relating to the rate of glycerol removal and for T½ values to be calculated. The T½ value did not appear to be meaningfully correlated to levels of fasting cholesterol or triglyceride. Mean disappearance rates were also calculated for control subjects, those with PVD and those who had sustained a myocardial infarction 3 weeks before. Values were similar for each group suggesting no abnormality of glycerol metabolism is present in subjects with vascular disease and that it does not occur as a consequence of myocardial infarction. The effect of glycerol on FFA levels was one of reduction (when measured by the correct method) and this could possibly be a consequence of gluconeogenesis. Results for insulin values are not available although it should be noted that Pelkonen, Miettinen, Taskinen and Nikkila (1968)[100] found that immunoreactive insulin levels did not change after oral administration of glycerol.

Studies were also done on rate of glycerol removal before and during cigarette smoking enhanced lipolysis. Smoking throughout the hour following glycerol administration did not significantly alter glycerol T½ values. The overall conclusion would be that rate of glycerol removal from the blood is not altered by underlying vascular disease and is unchanged by the acute effect of cigarette smoking.

CONCLUSIONS

1. Cigarette smoking following a recent myocardial infarction is associated with an enhanced release of FFA and ketone bodies. A similar enhanced response is found after subcutaneous adrenaline.
2. Subjects with stable vascular disease (PVD or CHD as manifest by angina) resemble control subjects in their response to cigarette smoking. This is so when either 2 or 6 cigarettes are smoked.
3. The enhanced response found after infarction is temporary rather than persistent. Subjects studied one year after infarction resemble control subjects in their response to smoking.
4. The beta-adrenoreceptor blocking agent, propranolol, abolishes the FFA response to smoking.
5. Smoking 2 cigarettes does not affect glucose, insulin, cholesterol or triglyceride values. Smoking 6 cigarettes produces a variable effect on glucose but only occasionally alters insulin levels.
6. The rate of removal of intravenously administered glycerol can be expressed as a single exponential. The values are similar in control and vascular disease subjects and are not affected by cigarette smoking.
7. Measurement of FFA levels by the Dole method after administration of glycerol produces a positive error, Possible explanations for this are discussed.

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